PHYLOGENETIC, PHENOTYPIC AND TRANSCRIPTOMIC CHARACTERIZATION OF READY-TO-EAT FOOD ASSOCIATED *LISTERIA MONOCYTOGENES*

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PHYLOGENETIC, PHENOTYPIC AND TRANSCRIPTOMIC CHARACTERIZATIONS OF READY-TO-EAT FOOD ASSOCIATED *LISTERIA MONOCYTOGENES*

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Listeria monocytogenes is of particular concern in ready-to-eat (RTE) food product. It is able to survive and grow in RTE foods from low numbers to potentially hazardous levels. With the aim of developing control strategies that prevent or reduce growth of this pathogen in RTE food products, I combined both applied and basic research approaches to perform phylogenetic, phenotypic and transcriptomic characterizations of RTE food associated *L. monocytogenes*, enabling a better understanding of *L. monocytogenes* population genetics, biological capacities, as well as the mechanisms that it uses to survive and grow in food matrices.

First, we used a 10-gene multilocus sequence typing scheme to investigate the diversity and phylogenetic distribution of 124 *L. monocytogenes* strains with various genetic backgrounds. Our data show that 10-gene MLST allows for high level of discrimination of *L. monocytogenes* and demonstrated the distinct ability of growth of *L. monocytogenes* under nutrient limited conditions between some of the subgroups of *L. monocytogenes.*

Second, we quantify the effects of organic acids, nisin, and their combinations on controlling 18 strains (individually) of *L. monocytogenes* growing on the RTE seafood, cold-smoked salmon (CSS), and in modified BHI broth (MBHIB) at 7^{o}C. The combination of potassium lactate and sodium diacetate, and the combination of potassium lactate and nisin, were identified as the most effective bacteriostatic treatment and bactericidal treatment against *L. monocytogenes*, respectively. We also observed that the quantitative prediction of the variability

of growth parameters in a food matrix such as CSS by using a laboratory medium could only be achieved for lineage II strains but not lineage I strains.

RNA-seq was used to understand the transcriptional landscape of *L. monocytogenes* strain H7858 grown on cold-smoked salmon (CSS, water-phase salt 4.65%, pH 6.1) relative to in modified brain heart infusion broth (MBHIB, water-phase salt 4.65%, pH 6.1) at 7^{o}C. We found that genes encoding proteins involved in cobalamin biosynthesis as well as ethanolamine and 1,2-propanediol utilization have significantly higher transcript levels in H7858 grown on CSS compared to in MBHIB. Our data identify specific transcriptional profiles of *L. monocytogenes* growing on vacuum-packaged CSS, which may provide targets for development of novel and improved strategies to control *L. monocytogenes* growth on this RTE food.

BIOGRAPHICAL SKETCH

Silin Tang was born on June 4th, 1985 in China. She graduated from Northeast Agricultural University in China with a bachelor's degree in Veterinary Medicine in 2009. Right in that year, several food safety issues emerged in China alarmed her that the food safety problem is a growing concern in the agricultural and food industry internationally, not just in her homeland. Because of this, she decided to pursue her Ph.D. degree in Food Science and Technology, mainly in Food Safety and Food Microbiology overseas, with her knowledge about livestock and microorganisms. Prior to her doctoral studies, Silin worked as a student intern at the Clinic for Poultry, University of Veterinary Medicine Hannover, Germany, with Dr. Ulrich Neumann and Dr. Silke Rautenschlein. She also worked as a student intern in the Preventive Veterinary Medicine Laboratory, College of Veterinary Medicine, Northeast Agricultural University, China, with Dr. Xiaofeng Ren and Dr. Junwei Wang. In 2009, Silin left China to join a Ph.D. program in the Laboratory for Food Microbiology & Pathogenesis of Foodborne Diseases at Cornell University under the supervision of Dr. Martin Wiedmann. At Cornell, She majored in Food Science, minored in Microbiology under the guidance of Dr. Randy W. Worobo, and in Applied Economics and Management under the guidance of Dr. Andrew M. Novakovic. Silin will begin a position as a post-doctoral researcher in Dr. Martin Wiedmann and Dr. Kathryn Boor's research group in April 2015.

Dedicated To The Ones I Love

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is an opportunistic foodborne pathogen that causes a severe life-threatening disease, listeriosis, particularly in neonates, pregnant women, elderly people and other immunologically-susceptible persons. It is estimated to cause approximately 1600 illnesses annually with a high case fatality rate of 20 ~30% in the United States (1). Characterization of *L. monocytogenes* isolates by multiple subtyping methods (2-4) has shown that strains can belong to one of four distinct genetic lineages (5, 6) with apparent differences in virulence (7, 8), growth under adverse conditions (9, 10) and gene expression related to virulence and stress response (11, 12). Lineage I strains are associated with most human listeriosis outbreaks, lineage II strains are predominant in foods, environments, animal listeriosis cases and sporadic human clinical cases, whereas lineage III and IV are mainly from animal sources (13). Among the 13 known serotypes of *L. monocytogenes,* three serotypes including 4b, belonging to lineage I, as well as 1/2a and 1/2b, belonging to lineage II, are most commonly associated with human illnesses (14). Using molecular typing method such as multilocus sequence typing to place *L. monocytogenes* isolates with various genetic backgrounds into clonal framework will facilitate a better understanding of (i) whether particular clusters of *L. monocytogenes* isolates are more virulent and have higher epidemic potential, and (ii) which phenotypic characters, if any, are linked to *L. monocytogenes* more likely to be associated with outbreaks than the others. In addition, these genetic variations of *L. monocytogenes* clearly illustrate the importance of using strains representing different genetic backgrounds in studies on *L. monocytogenes* stress survival and virulence as well as in validation studies for *L. monocytogenes* control strategies.

L. monocytogenes is ubiquitously prevalent in the environment and almost exclusively transmitted by food (15, 16). It has been identified as of particular concern to the ready-to-eat (RTE)-meat and -seafood industries due to its ability to grow at temperatures as low as -0.4^{o}C and salt contents as high as 25% (at 4^{o}C) (17-19), which elevate the risk of food contamination and transmission of this pathogen through RTE foods to humans. Cold smoked salmon (CSS), a RTE seafood, represents a typical food product that can support the growth of *L. monocytogenes* from low numbers to potentially hazardous levels (20-25). Consequently, this product has been identified as having a high risk per serving for listeriosis (22, 23). The heat treatment applied during processing of CSS, as well as the typical product characteristics of CSS including pH, water activity, salt, sodium nitrite, and phenolic components are not sufficient to control the growth of *L. monocytogenes* (24, 25). Therefore, it is critical to develop control strategies that prevent or reduce growth of this pathogen in RTE food including the RTE seafood cold-smoked salmon.

One potential method to control *L. monocytogenes* in RTE food is the use of bactericidal or bacteriostatic antimicrobial agents, such as nisin, potassium lactate and sodium diacetate. Nisin (NI), a bacteriocin produced by *Lactococcus lactis*, is known to reduce the population of *L. monocytogenes* in various food products, including cold-smoked salmon (26-29). Salts of organic acids, such as potassium lactate (PL) and sodium diacetate (SDA), are also widely used in the RTE meat industry as antimicrobial agents (30), and have also been evaluated for use in CSS (23, 31). Meanwhile, combining antimicrobial agents in a food system can often enhance antimicrobial action against pathogenic and/or spoilage organisms (32-34). As challenge studies in food matrices can be costly and time-consuming, there is potential to utilize antimicrobial efficacy data generated from studies in laboratory medium to predict efficacy in food matrices.

Strong correlations would allow data generated from laboratory medium to be used as an effective and rapid initial screen for antimicrobial efficacy.

On the other hand, with the aim of developing control strategies that prevent or reduce growth of this pathogen in RTE seafood products, there is a need for better understanding of the mechanisms that *L. monocytogenes* uses to survive and grow in food matrices. Changes in gene expression in response to various environments can be used to determine the physiological state of *L. monocytogenes* under different conditions, and identify the metabolic pathways that are important for survival and growth of *L. monocytogenes* in food products. This will allow the identification of new compounds that could specifically interfere with these metabolic pathways and thereby control the growth of *L. monocytogenes* (35). However, the majority of data from previous studies are based on exposure of *L. monocytogenes* to specific stresses in laboratory medium, providing information about specific stress responses in a controlled environment. This information may not provide the full extent of stress responses in a more complex environment, such as a food matrix, as a previous study has demonstrated that *L. monocytogenes* may react differently to stress in a food matrix (e.g., growth on CSS) as compared to in a laboratory setting (e.g., growth in brain heart infusion broth), especially for lineage I strains of *L. monocytogenes* (36), indicating the importance of the investigation of *L. monocytogenes* transcriptome in a real food matrix.

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CHAPTER 2

CLONAL CLUSTERING USING 10-GENE MULTILOCUS SEQUENCE TYPING REVEALS AN ASSOCIATION BETWEEN GENOTYPE AND *LISTERIA MONOCYTOGENES* MAXIMUM GROWTH RATE IN DEFINED MEDIUM

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ABSTRACT

We used a 10-gene multilocus sequence typing (10G-MLST) scheme to investigate the diversity and phylogenetic distribution of 124 *L. monocytogenes* strains across major lineages, major serotypes, and seven epidemic clones (ECs) that have been previously associated with outbreaks. The 124 isolates proved to be diverse, with a total of 81 sequence types (10G-STs) belonging to 13 clonal complexes (CCs), where all STs of the same CC differ from one another in up to three of the ten alleles (named as 10G-triple-locus-variant-clonal-complexes [10G-TLV-CCs]). Phenotypic characterization for 105 of the 124 strains showed that *L. monocytogenes* had variable maximum growth rate (μ_{max}) in a defined medium at 16^{o}C, and classification by lineage or serotype was not able to reflect the genetic basis for the difference of this phenotype. Among the six major 10G-TLV-CCs, 10G-TLV-CC4 that included lineage I strains had significantly lower μ_{max} (Tukey HSD adjusted (adj.) $P < 0.05$) compared to 10G-TLV-CC1 and 10G-TLV-CC3 that both comprised lineage II strains, indicating a distinct difference in growth of these *L. monocytogenes* isolates under nutrient limited conditions among some of the CCs. However, the

other three (10G-TLV-CC2, 6 and 10) of the six major 10G-TLV-CCs containing either lineage I or lineage II strains did not show significantly different μ_{max} compared to the others (adj. $P < 0.05$). Our findings highlighted the importance of using molecular typing methods that can be used in evolutionary analyses as a framework for further understanding the phenotypic characteristics of subgroups of *L. monocytogenes*.

INTRODUCTION

Listeria monocytogenes is an opportunistic foodborne pathogen that poses a particular risk for pregnant women, neonates, elderly people and other immunologically-susceptible persons. It is ubiquitous in the environment and almost exclusively transmitted by food (1, 2). *L. monocytogenes* isolates have been differentiated into four genetic lineages (I ~ IV) (3, 4) and 13 known serotypes (5). Despite having similar prevalence in food and environment samples, lineage I and II strains are more likely to be associated with human hosts and non-human (e.g., animal, food and environment) sources, respectively (6), whereas lineage III and IV are mainly from animal hosts (3). Serotypes 4b, 1/2a and 1/2b are most commonly associated with human illnesses (5). Phenotypic characterization of *L. monocytogenes* isolates has shown apparent differences among lineages and serotypes in virulence (7, 8) and growth under various conditions (9-11). However, with the aim of developing control strategies that prevent *L. monocytogenes* outbreaks, further study is needed to investigate (i) whether particular genetic subtypes of *L. monocytogenes* are more persistent in environment or food, potentially increasing human exposure to these strains, and (ii) which phenotypic characteristics, if any, are linked to *L. monocytogenes* that are more likely to be associated with outbreaks.

Multi-virulence-locus sequence typing (MvLST) (12, 13) together with pulsed-field gel electrophoresis (PFGE) (14), have been used to define epidemic clones (ECs) of *L. monocytogenes*, where isolates that are genetically related but implicated in temporally- and geographically-distinct outbreaks are classified in one EC (12, 15-17). Multilocus sequence typing (MLST) (18) which targets seven housekeeping genes (7G-MLST) has been used to define the clonal complexes (CCs) of *L. monocytogenes* (19, 20). Cantinelli et al. (21) recently

confirmed the correspondence between ECs and CCs in terms of phylogenetic clustering and discriminatory power. In this study, we used 10-gene MLST (10G-MLST) (22) to investigate the phylogenetic diversity of *L. monocytogenes* strains. Further, we compared the available classification results based on these previously described typing schemes (CCs and ECs identified by using 7G-MLST and MvLST scheme, respectively) to the one we used (10G-MLST scheme), to understand the correlations among them and enable cross-referencing between the phylogenetic classification results of these schemes.

The ability to persist at low temperatures and in nutrient limited food processing environments elevates the risk of food contamination and transmission of this pathogen through foods to humans (23, 24), thus possibly representing one of the important phenotypes to identify the *L. monocytogenes* isolates with greater potential to spread through the food supply. Since Lineage III and VI of *L. monocytogenes* are rarely isolated from human related cases or from food, we focused on Lineage I and II, and compared the maximum growth rates (μ_{max}) of *L. monocytogenes* isolates growing in a chemically defined medium (DM) at 16^{o}C by lineage (I & II) and 10G-MLST defined CCs (where all STs of the same CC differ from one another in three or fewer of the ten alleles). This will facilitate better understanding of the correlation between phenotype and genotype of *L. monocytogenes.*

MATERIALS AND METHODS

Isolates. For measurement of μ_{max} in DM, a group of 105 isolates was assembled from our collection [the Cornell University Food Safety Laboratory (CUFSL)] (Table S2.1) to represent isolates from a wide range of sources (Table 2.1, 2.2) and diverse genetic backgrounds based on ribotype data (Table S2.1). For 10G-MLST analysis, we (i) sequenced the MLST genes

for 89 of the 105 isolates, and (ii) extracted MLST gene sequences for the remaining 16 isolates from the public sequence repositories. Second, in addition to these 105 isolates, 19 isolates with full genome sequence data available were included in the 10G-MLST analysis by extracting MLST gene sequences from the public sequence repositories (Table S2.1). The whole isolate set included (i) 24 human and food isolates from 15 outbreaks, (ii) 64 human and food isolates from non-outbreak cases, (iii) 12 animal isolates, and (iv) 18 environmental isolates, for a total of 124 *L. monocytogenes* isolates representing lineages I, II, III, and IV, as well as seven of the ECs.

Table 2.1. Lineage I isolates used for 10-gene multilocus sequence typing (10G-MLST) analysis

Strain ID[a]	Source	10G-ST[b]	10G-SLV-CC[c]	10G-TLV-CC[d]	7G-ST[e]	7G-CC[f]	μ_{max}[g] [log(OD600/ml)/day]
FSL J1-0220	1979, human, Boston vegetable outbreak	2	2	2	NA[h]	NA	0.047
FSL J1-0225	1983, human, Massachusetts milk outbreak	9	2	2	290	2	0.058
FSL R2-0583	1983, human, Massachusetts milk outbreak	9	2	2	NA	2	0.039
FSL R2-0578	1983, human, Massachusetts milk outbreak	38	2	2	NA	2	0.05
FSL J1-0020	1987, human, Philadelphia multiple foods outbreak	9	2	2	NA	2	0.052
FSL J1-0129	1988-1989, human, UK pate outbreak	2	2	2	NA	2	0.046
FSL R2-0585	1988-1990, food, UK pate outbreak	2	2	2	NA	2	0.021
FSL R2-0589	1988-1990, food, UK pate outbreak	36	2	2	NA	2	0.041
FSL J1-0116	1988-1990, human, UK pate outbreak	2	2	2	2	2	0.062
HPB2262	1997, Italian gastroenteritis outbreak	2	2	2	2	2	NA
FSL F2-0091	1999, human, sporadic	2	2	2	NA	NA	0.055
FSL M2-0042	1999, human, sporadic	37	2	2	NA	NA	0.041
FSL F2-0656	2001, human, sporadic	2	2	2	NA	NA	0.041
FSL F2-0661	2001, human, sporadic	2	2	2	NA	NA	0.041
FSL S4-0848	2002, environment (sidewalk floor)	39	2	2	2	2	0.034
ATCC 19117	animal (sheep), sporadic	2	2	2	2	2	NA
SLCC 2755	1967, animal (chinchilla)	47	5	4	66	3	NA[h]
FSL R2-0502	1994, food, Illinois chocolate milk outbreak	1	5	4	3	3	0.04
FSL R2-0598	1994, human, Illinois chocolate milk outbreak	1	5	4	NA	3	0.06
FSL N1-0017	1998, food	21	5	4	3	3	0.033
FSL M1-0006	1998, human, sporadic	19	S[i]	4	NA	NA	0.012
FSL F2-0369	2000, food (RTE pasta salad)	1	5	4	NA	NA	0.043
FSL R2-0154	2001, food (smoked seafood)	1	5	4	NA	3	0.055
FSL L3-0051	2002,food (RTE salmon)	1	5	4	NA	3	0.034
FSL F6-0386	2007, food (smoked salmon)	20	5	4	NA	NA	0.049
FSL J1-0049	human sporadic	1	5	4	3	3	0.028
FSL J1-0194	1997, human, sporadic	30	3	5	88	S	0.036
FSL F2-0521	2000, food (smoked fish salad)	31	3	5	NA	NA	0.048
FSL R8-5459	2010, environment (floor)	32	3	5	NA	NA	0.05
FSL N1-0260	human	33	3	5	NA	NA	0.031
FSL R2-0557	human, sporadic	34	3	5	NA	NA	0.033
FSL F6-0366	1998-1999, food (meat), US hot dog outbreak	4	6	6	6	6	0.034
FSL N1-0225	1998-1999, human, US hot dog outbreak	29	6	6	6	6	0.05
FSL N1-0061	1998, food (salmon brine)	27	6	6	NA	NA	0.053

Table 2.1 (Continued)

FSL E1-0201	2001, environment (soil)	4	6	6	NA	NA	0.053
FSL N3-0780	2002, animal (bovine feces)	4	6	6	6	6	0.055
FSL N3-0692	2002, environment (soil)	4	6	6	6	6	0.054
FSL R2-0763	2002, human, Northeastern states sliced deli meat outbreak	4	6	6	6	6	0.044
FSL F3-0950	2005, human, sporadic	4	6	6	NA	6	0.049
FSL F6-0095	2005, human, sporadic	28	6	6	NA	NA	0.055
FSL F2-0699	2002, human, sporadic	14	7	7	NA	NA	0.049
FSL F3-0757	2002, human, sporadic	14	7	7	NA	NA	0.028
FSL H5-0804	2006, environment (deli floor drain)	59	7	7	NA	NA	0.052
FSL J1-0175	environment (water)	58	7	7	87	S	0.052
FSL J1-0108	1981, human, Halifax coleslaw outbreak	8	8	8	1	1	0.059
FSL J1-0126	1983-1987, human, Switzerland cheese outbreak	8	8	8	1	1	0.033
FSL J1-0110	1985, food, Mexican-style cheese outbreak	8	8	8	1	1	0.052
FSL C1-0122	1998, human, sporadic	22	8	8	1	1	0.043
SLCC 2378	poultry	45	8	8	73	1	NA
FSL J2-0045	1992, animal (sheep), clinical	25	10	9	NA	NA	0.044
FSL F2-0018	1999, human, sporadic	24	S	9	NA	NA	0.047
FSL F2-0366	2000, human, sporadic	26	10	9	NA	NA	0.042
FSL S4-0643	2002, environment (bench)	11	9	12	NA	4	0.052
L312	food (cheese)	11	9	12	4	4	NA
07PF0776	human (myocardial abscess), sporadic	42	9	12	4	4	NA
FSL J1-0169	1996, human, sporadic	7	S	S[i]	5	5	0.051
FSL E1-0041	2000, animal (sheep), clinical	41	S	S	NA	NA	0.051
FSL F2-0493	2000, food (raw chunk beef patties)	35	S	S	NA	NA	0.047
FSL R2-0501	2000, human, North Carolina cheese outbreak	40	S	S	558	S	0.043
FSL R2-0182	2001, food (smoked seafood)	7	S	S	NA	5	0.061
FSL F2-0693	2001, human, sporadic	60	S	S	NA	NA	0.036
FSL S4-0440	2002, environment (sidewalk floor)	23	S	S	379	NA	0.049
FSL S10-630	2010, environment (produce farm)	43	S	S	NA	NA	0.048
SLCC 2540	1956, human, sporadic	46	S	S	617	3	NA

[a]Isolates are listed in numerical order of the "Lineage", and then in "10G-TLV-CC" .
[b]10G-ST, sequence type (ST) identified by using 10G-MLST scheme.
[c]10G-SLV-CC, single-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the clonal complex (CC) differ from one another in one of the ten alleles.
[d]10G-TLV-CC, triple-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the same CC differ from one another in no more than three of the ten alleles.
[e]7G-ST, ST identified by using 7-gene multilocus sequence typing (7G-MLST) scheme.

[f]7G-CC, CC identified by using 7G-MLST scheme, where all ST in the same CC differ from one another in one of the seven alleles.
[g]μ_{max}, the growth parameter, maximum growth rate, of *L. monocytogenes* isolates growing in defined medium (DM) at 16^{o}C.
[h]NA, not available.
[i]S, singleton, (i) for 10G-SLV-CCs and 10G-TLV-CCs, singletons were defined as STs differing from all of the other STs in more than one or more than three of the ten alleles, respectively, (ii) for 7G-CCs, singletons were defined as STs differing from all of the other STs in more than one of the seven alleles.

Table 2.2. Lineage II isolates used for 10-gene multilocus sequence typing (10G-MLST) analysis

Strain ID[a]	Source	10G-ST[b]	10G-SLV-CC[c]	10G-TLV-CC[d]	7G-ST[e]	7G-CC[f]	μ_{max}[g] [log(OD 600/ml)/ day]
FSL R2-0559	1998, food, sporadic	5	1	1	11	11	0.054
FSL J1-0101	1998, human, sporadic	5	1	1	86	11	0.04
FSL C1-0111	1998, human, sporadic	77	S[h]	1	NA[i]	NA	0.037
FSL F2-0237	1999, food (smoked salmon)	18	S	1	NA	NA	0.045
FSL F2-0141	1999, human, sporadic	5	1	1	NA	NA	0.055
FSL F2-0048	1999, human, sporadic	70	1	1	NA	NA	0.063
FSL R2-0011	2000, food (RTE deli salad)	16	1	1	NA	NA	0.057
FSL F6-0154	2000, food, US turkey deli meat outbreak	5	1	1	86	11	0.053
FSL F2-0516	2000, human, sporadic	16	1	1	NA	NA	0.02
FSL F2-0405	2000, human, sporadic	71	1	1	NA	NA	0.054
FSL R2-0499	2000, human, US turkey deli meat outbreak	76	1	1	11	11	0.059
FSL T1-0073	2001, food (raw Norweigan)	81	S	1	NA	NA	0.065
FSL R2-0487	2001, food (RTE bagged salad)	74	1	1	NA	NA	0.059
FSL L3-0123	2002, environment (floor drain)	72	1	1	NA	NA	0.061
FSL F3-0566	2003, human, sporadic	73	1	1	NA	NA	0.066
FSL F3-0995	2006, human, sporadic	18	S	1	NA	NA	0.055
FSL R8-0879	2008, environment (floor drain)	75	S	1	NA	NA	0.061
FSL X1-0001	1987, human, sporadic	69	4	3	85	7	0.047
FSL F2-0194	1999, human, sporadic	68	S	3	7	7	0.063
FSL L4-0100	2002, environment (fish processing floor mat)	49	4	3	NA	NA	0.059
FSL L4-0096	2002, environment (fish processing plant drain)	50	4	3	NA	NA	0.059
FSL F3-0744	2002, human, sporadic	3	4	3	NA	NA	0.058
FSL F3-0631	2003, human, sporadic	3	4	3	NA	NA	0.048
FSL F6-0084	2004, human, sporadic	3	4	3	NA	NA	0.055
FSL R6-0896	2007, environment (floor drain)	3	4	3	NA	7	0.064
SLCC 5850	1924, animal (rabbit)	3	4	3	12	7	NA
FSL F2-0539	1924 animal (rabbit)	6	11	10	35	9	0.044
SLCC 2372	1935, human, sporadic	13	11	10	122	9	NA
SLCC 2479	1966, human, sporadic	6	11	10	9	9	NA
FSL J1-0022	human sporadic	13	11	10	NA	9	0.052
FSL G2-0003	human, reference strain	61	S	10	210	9	0.034
FSL J1-0125	human, sporadic	6	11	10	NA	9	0.039
FSL R2-0561	human, sporadic	6	11	10	9	9	0.053
FSL R2-0089	2000, food (RTE deli salad)	62	12	13	NA	NA	0.047
FSL F2-0515	2000, food (RTE turkey deli meat)	15	12	13	NA	NA	0.039
FSL L4-0151	2002, environment (raw salmon room floor drain)	15	12	13	NA	NA	0.044

Table 2.2 (Continued)

SLCC 7179	1986, food (cheese)	51	S	S	91	NA	NA
FSL J2-0003	1993, animal (bovine), clinical	79	S	S	89	NA	0.048
FSL J2-0054	1993, animal (sheep), clinical	48	S	S	412	S	0.061
Finland1998	1998, Finland butter outbreak	10	S	S	155	NA	NA
FSL C1-0051	1998, human sporadic	10	S	S	NA	NA	0.054
FSL C1-0115	1998, human, sporadic	80	S	S	370	S	0.047
FSL F2-0032	1999, food (smoked whitefish)	66	S	S	NA	NA	0.054
FSL F2-0216	1999, human, epidemic	10	S	S	NA	NA	0.054
FSL F2-0039	1999, human, sporadic	63	S	S	NA	NA	0.049
FSL E1-0154	2001, environment (soil)	65	S	S	NA	NA	0.048
FSL R2-0493	2001, food (RTE deli salad)	64	S	S	NA	NA	0.018
FSL L4-0396	2002, environment (finished product area drain)	67	S	S	NA	NA	0.048
FSL N3-0165	2002, environment (soil)	78	S	S	90	90	0.057
08_5578	2008, Canadian deli meat outbreak	17	S	S	292	8	NA
08_9523	2008, Canadian deli meat outbreak	17	S	S	120	8	NA

[a]Isolates are listed in numerical order of the "Lineage", and then in "10G-TLV-CC" .
[b]10G-ST, sequence type (ST) identified by using 10G-MLST scheme.
[c]10G-SLV-CC, single-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the clonal complex (CC) differ from one another in one of the ten alleles.
[d]10G-TLV-CC, triple-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the same CC differ from one another in no more than three of the ten alleles.
[e]7G-ST, ST identified by using 7-gene multilocus sequence typing (7G-MLST) scheme.
[f]7G-CC, CC identified by using 7G-MLST scheme, where all ST in the same CC differ from one another in one of the seven alleles.
[g]μ_{max}, the growth parameter, maximum growth rate, of *L. monocytogenes* isolates growing in defined medium (DM) at 16^{o}C.
[h]S, singleton, (i) for 10G-SLV-CCs and 10G-TLV-CCs, singletons were defined as STs differing from all of the other STs in more than one or more than three of the ten alleles, respectively, (ii) for 7G-CCs, singletons were defined as STs differing from all of the other STs in more than one of the seven alleles.
[i]NA, not available

MLST. All of the 124 isolates were characterized using a previously described 10G-MLST scheme (22), which includes DNA sequences of partial open reading frames for ten genes: *ldh*, *lmo0490*, *prs*, *sigB*, *polC*, *rarA*, *lmo1555*, *pbpA*, *addB*, and *lmo2763* (22). This method was selected as it was designed to be used for the majority of *Listeria* species, not just *monocytogenes,* and has the potential to be more discriminatory with the inclusion of 10 genes rather than 7. The 10G-MLST data for the isolates from our collection (CUFSL, Table S2.1) can be found on Food Microbe Tracker (http://www.foodmicrobetracker.com). Sequence types based on 10G-MLST (10G-STs) (Table 2.1, 2.2) were determined by using DnaSP v.5 (25). For each 10G-MLST locus, an allele number was given to each distinct sequence variant, and a distinct 10G-ST number was attributed to each distinct combination of alleles at the ten genes. 10G-ST numbers were initially based on highest frequency for the 10G-STs, and were subsequently increased arbitrarily.

The eBURST V3 (26) algorithm was used for the classification of single-locus-variant-clonal complexes (10G-SLV-CCs) and triple-locus-variant-clonal complexes (10G-TLV-CCs). All 10G-STs assigned to the same 10G-SLV-CC and 10G-TLV-CC share identical alleles at nine and seven of ten loci, respectively. Singletons were accordingly defined as 10G-STs having at least two and four allelic mismatches with all other 10G-STs (Fig S2.1, Table 2.1, 2.2) for 10G-SLV-CCs or 10G-TLV-CCs, respectively. The central 10G-ST of a clonal complex (CC) was defined as the ST that differed from the largest number of other STs at only a single locus or at three loci for 10G-SLV-CCs or 10G-TV-CCs, respectively. The goeBURST 1.2.1 (27) algorithm was used to visualize the relationship of the 10G-STs by generating a full minimum spinning tree with PHYLOViZ (www.phyloviz.net) (Fig. 2.1).

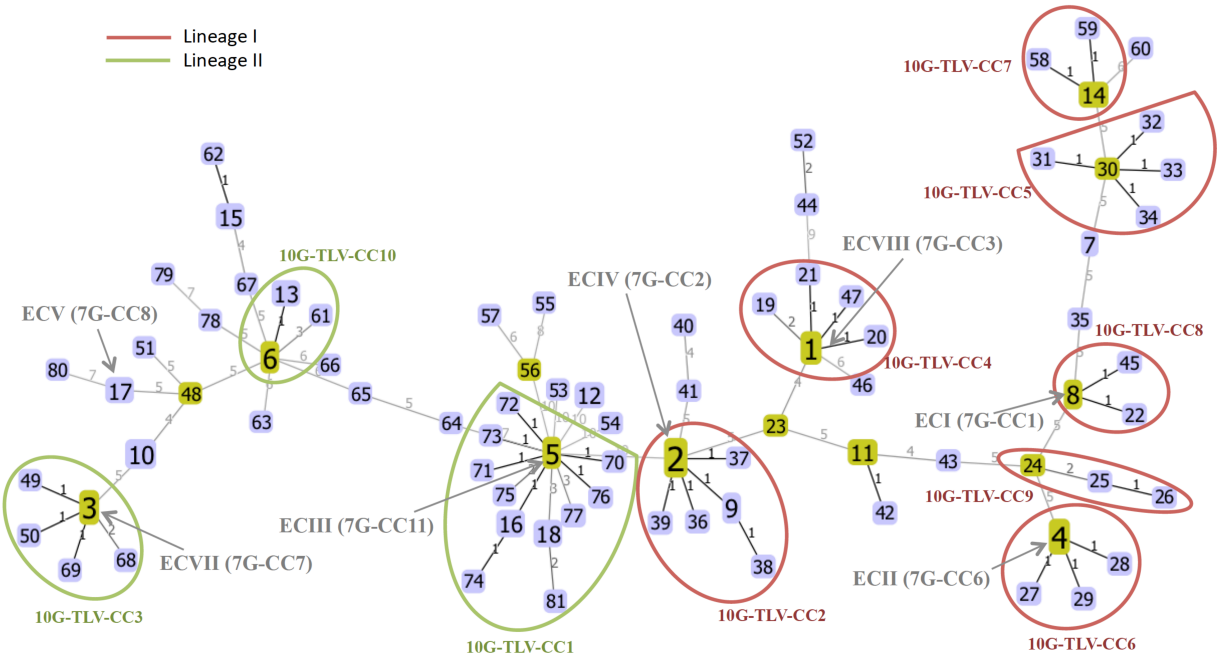

Figure 2.1. Ten major 10G-TLV-CCs and the minimum spanning tree generated by goeBURST for all 10G-STs

Ten major 10G-TLV-CCs and the minimum spanning tree generated by goeBURST for all 10G-STs. 10G-TLV-CCs were identified by using 10G-MLST scheme and eBURST algorithm, where all 10G-STs of the same 10G-TLV-CC differ one another in no more than three of the ten alleles. Ten major 10G-TLV-CCs (defined as the 10G-TLV-CCs containing more than 2 10G-STs) are depicted by red (lineage I) and green (lineage II) circles. Seven ECs and the 7G-CCs are designated next to their corresponding 10G-TLV-CCs and specifically pointed to the group founder 10G-STs. The identified group founder 10G-STs are represented by light-green elements, and the other 10G-STs are represented by blue. The size of each element reflects the number of isolates within a 10G-ST. Locus variant levels (level 1-10) are shown on links between 10G-STs.

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For 60 of the 124 isolates, CC (defined as 7G-CC in present study) were assigned to each strain based on sequence types (ST) (defined as 7G-ST in present study) (Table 2.1, 2.2) presented in the Pasteur MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html) and in the studies of Ragon et al. (20) and Cantinelli et al. (21). All 7G-STs assigned to the same 7G-CC share identical alleles at six of seven loci, and singletons were accordingly defined as 7G-STs having at least two allelic mismatches with all other 7G-STs (Table 2.1, 2.2). This scheme includes DNA sequences of partial open reading frames for seven genes including *abcZ, bglA, cat, dapE, dat, ldh,* and *lhkA* (20). There is one gene *(ldh*) in common between the 7G-MLST and10G-MLST scheme.

Maximum growth rate in defined media. To simulate a nutrient-limited (e.g., food processing) environment for *L. monocytogenes* growth, we measured μ_{max} of 105 *L. monocytogenes* isolates using a DM specific to *L. monocytogenes* (28) at 16^{o}C, with 25 mM glucose as the carbon source. For growth prior to inoculation in DM, we followed the approach previously described by Stasiewicz et al. (29). In the DM, OD_{600} readings were taken from hour 0~3 as time point 0 (started from the first detectable OD_{600} value of each sample), and at every 6 or 12 hours after time point 0 until stationary phase (up to 96 hours). The four-factor modified logistic growth model described by Baranyi and Roberts (30) was used to calculate the growth parameter μ_{max}, [log(OD_{600}/ml)/day], for each strain. Regression was carried out by using the NLStools package (v 0.0-5) in R v2.6.2. For growth in DM, each strain was tested once; multiple strains per CC were considered replicates.

Association of growth rates. One-way analysis of variance (ANOVA) were used to determine if μ_{max} was significantly different between lineages and among 10G-TLV-CCs for the

105 strains tested (Fig. 2.1), where the values of the μ_{max} served as the response. The linear model used for the ANOVA was: $Y(\mu_{max})$ = lineage or 10G-TLV-CC + strain + E(error). Strain was treated as a random effect. Significant difference between a given pair of lineages or 10G-TLV-CCs was identified by the Student's T test and Tukey HSD method ($\alpha = 0.05$; JMP 7, SAS Institute, Inc., Cary, NC), respectively. Student's T test p-value (P) and Tukey HSD adjusted p-values (adj. P) are reported as significant when < 0.05.

RESULTS AND DISCUSSION

Ten-gene MLST allows for a high level of discrimination of *L. monocytogenes.* Based on 10G-MLST, the 124 isolates were found to represent a total of 81 10G-STs (Fig S2.1, Table 2.1, 2.2). Four 10G-STs, 10G-ST1, 2, 3, and 4 predominated, with five or more isolates belonging to each of the 4 STs, and cumulatively account for 21% isolates (26/124). Among them, 10G-ST1, 2 and 4 belong to lineage I, and 10G-ST3 belongs to lineage II. Overall, lineage I (39 STs, 64 isolates) contains more STs than lineage II (34 STs, 51 isolates). All 10G-STs containing more than one isolate originated from more than one source, which is consistent with the finding of Haase et al. (6). The 10G-STs, 10G-ST1, 2 and 3, all included outbreak related isolates and correspond to 7G-ST3, 7G-ST2, and 7G-ST6, respectively. Interestingly, despite that two isolates from the US hot dog outbreak FSL F6-0366 (H7858) and FSL N1-0225 (H7550) were both assigned as 7G-ST6 previously, they were differentiated into two distinct 10G-STs (10G-ST4 and 10G-ST29). The 10G-MLST showed a higher discriminatory power (D = 0.9842) (31) compared to 7G-MLST (D= 0.9774), indicating that, although with some exceptions, 10G-MLST is generally more discriminatory than 7G-MLST (Table 2.1, 2.2). This suggests that 10G-MLST is an effective approach for characterizing *L. monocytogenes* DNA sequence

polymorphism with a higher resolution compared to the 7G-MLST scheme.

While 10G-MLST shows increased discrimination over 7G-MLST, most likely these methods will be replaced with whole genome sequencing (WGS) (32), which is being adopted by public health agencies for routine surveillance (33, 34), including an MLST-based approach to analyze genome sequence data (wgMLST) (35). However, interpreting genetic variation will present new challenges to investigation using WGS or wgMLST, whereas the use of smaller number of genes for clustering may still be useful and efficient to define genetically distinct groups with phenotypic differences. In addition, 7G- or 10G-MLST data will be easily comparable with genome based data, since the necessary sequences can be easily extracted from WGS data.

eBURST clustered 124 isolates into 12 10G-SLV-CCs and 13 10G-TLV-CCs. We used the eBURST algorithm to identify 12 10G-SLV-CCs and 42 singletons from 81 10G-STs, where all 10G-STs belonging to the same 10G-SLV-CCs share identical alleles at nine of the ten loci. Two 10G-SLV-CCs, 10G-SLV-CC1 (12 isolates, 8 STs) and 10G-SLV-CC2 (16 isolates, 6 STs), predominated and accumulatively accounted for 23% isolates (28/124), and 17% of the 10G-STs (14/81); they belong to lineage II and lineage I, respectively. Overall, lineage I (8 10G-SLV-CCs, 11 singletons) contains more 10G-SLV-CCs than lineage II (4 10G-SLV-CCs, 22 singletons). A large proportion of the outbreak-associated isolates were classified into 10G-SLV-CC2, accounting for 42% of the outbreak-associated isolates (10/24). Overall, isolate classifications into 10G-SLV-CCs and 7G-CCs were consistent for the majority of the clonal complexes (Table 2.1, 2.2) with some exceptions, for instance, isolates FSL J1-0169 and FSL R2-0182 assigned as 7G-CC5 were differentiated by 10G-MLST into two singletons.

A less stringent approach allowing three-locus variants between members identified 13 10G-TLV-CCs and 31 singletons among the 124 isolates (Table 2.1, 2.2). This approach allowed more isolates to be clustered into CCs, and reduced the number of singletons, facilitating a better understanding of the relationship between isolates. Among the 13 10G-TLV-CCs, ten CCs that each contain more than two 10G-STs were highlighted in Fig. 2.1, and account for 69% isolates (85/124) and 60% of 10G-STs (49/81) (Table 2.1, 2.2). The 26 outbreak-associated isolates were distributed in six of the 13 distinct 10G-TLV-CCs, including five of the 10G-TLV-CCs that each represent one EC (Fig. 2.1, Table S2.1). Among them, each of the ECs only reciprocally corresponds to one 10G-TLV-CCs , showing a consistency between the classification into ECs and 10G-TLV-CCs, which is similar to the findings of Cantinelli et al. (21) that MLST based CCs correspond to the EC classification.

Growth rates show considerable differences between lineages and between 10G-TLV-CCs. Our data showed that *L. monocytogenes* had highly variable μ_{max} across the 105 isolates in DM at 16^{o}C, ranging from 0.012 to 0.066 log(OD_{600}/ml)/day (Table 2.1, 2.2). More specifically, a one-way ANOVA model with lineage effect (two levels: lineage I and II) for the growth parameter μ_{max} showed a significant effect (P = 0.0059) of lineage on μ_{max} (Table 2.3). Lineage I isolates had significantly lower (P < 0.05) average μ_{max} [0.045 ± 0.010 log(OD_{600}/ml)/day] compared to lineage II isolates [0.052 ± 0.011 log(OD_{600}/ml)/day] (Table 2.3). In contrast, Bergholz et al. (10) demonstrated that lineage I strains had significantly higher growth rate than lineage II strains in brain heart infusion broth (BHIB) with 6% salt at 37^{o}C. Moreover, our previous study on *L. monocytogenes* growth on cold smoked salmon and in modified BHIB with 4.65% salt at 7^{o}C showed that, there was no significant difference between the average μ_{max} of lineage I and lineage II strains under either of the growth conditions (11).

Possible explanations for these observed differences include the high salt content and the difference of the incubation temperatures, while the DM used in the present study simulated a nutrient limited food-processing environment, usually with relatively low temperatures (about 16^{o}C). Similarly to our finding, Bruhn et al. (36) found that lineage II strains outcompeted lineage I strains in selective enrichment media. However, based on the findings of Haase et al. (6) that environmental and food isolates are equally distributed between lineage I and II, it is not likely that the ability of lineage II strains to grow faster in non-host environments (especially nutrient limited food processing environments) can increase the prevalence of all lineage II strains in environmental and food samples. Our observation, combined with the findings of Haase et al. (6), may suggest that it is more likely that just some of the subgroups of lineage II strains can grow faster than lineage I strains in such environments.

Table 2.3. Lineage-specific effects and 10G-TLV-CC-specific effects on maximum growth rate of *L. monocytogenes* isolates grown in defined medium at 16°C

Lineage (no. isolates)	μ_{max}[a] [log(OD600/ml)/day]	10G-TLV-CC[b] (no. isolates)	μ_{max} [log(OD600/ml)/day]
Lineage I (57)	0.045 ± 0.010[c] A[d]	CC2 (14)	0.046 ± 0.011 AB
		CC4 (9)	0.040 ± 0.015 B
		CC6 (9)	0.050 ± 0.007 AB
Lineage II (44)	0.052 ± 0.011 B	CC1 (17)	0.054 ± 0.012 A
		CC3 (8)	0.057 ± 0.006 A
		CC10 (5)	0.045 ± 0.009 AB

[a]μ_{max}, the growth parameter, maximum growth rate, of *L. monocytogenes* isolates growing in defined media (DM) at 16°C.
[b]10G-TLV-CC**,** triple-locus-variant-clonal complex identified by using 10-gene multilocus sequence typing (10G-MLST) scheme and eBURST algorithm, where all sequence types in the same clonal complex (CC) differ from one another in no more than three of the ten alleles.
[c]Results are summarized by means ± standard deviations for strains of each category, each strain was tested once.
[d]Means within a given column with the same letter are not statistically different from each other (overall $\alpha = 0.05$, Tukey's correction).

To further compare the growth rate of subgroups of lineage I and lineage II strains, a one-way ANOVA model with 10G-TLV-CC effect (six levels: 10G-TLV-CC1, 2, 3, 4, 6, 10, each having at least five isolates) for the growth parameter μ_{max} was conducted, and showed significant effect ($P = 0.0087$) of 10G-TLV-CCs on μ_{max} (Fig. 2.2, Table 2.3). The 10G-TLV-CC4 showed the lowest average μ_{max} (0.040 ± 0.015 $\log(OD_{600}/ml)/day$) and was significantly lower (adj. $P < 0.05$) than 10G-TLV-CC1 (0.053 ± 0.011 $\log(OD_{600}/ml)/day$) and 10G-TLV-CC3 (0.057 ± 0.006 $\log(OD_{600}/ml)/day$) (Fig. 2.2, Table 2.3). The average μ_{max} of each of the 10G-TLV-CC2 (7G-CC2), 10G-TLV-CC 6 (7G-CC6) and 10G-TLV-CC10 (7G-CC9) was not significantly different (adj. $P > 0.05$) from the others (Fig. 2.2, Table 2.3). The 10G-TLV-CC4, representing partial lineage I strains, corresponds to 7G-CC3. The 10G-TLV-C1 and 10G-TLV-C3, representing partial lineage II strains, correspond to 7G-CC11 and 7G-CC7 (Table S2.1). Interestingly, it has been reported that 7G-CC3 (10G-TLV-CC4) ranked among the 4 most common clones (7G-CC2, 7G-CC1, 7G-CC3, 7G-CC9) in the world, and is one of the most highly prevalent 7G-CCs (7G-CC1, 7G-CC2 and 7G-CC3) in lineage I (Chenal-Francisque et al., 2011). According to our result, it seems that the majority of the most prevalent 7G-CCs aforementioned, do not have a significant higher growth rate in DM as compared to the less prevalent 7G-CCs.

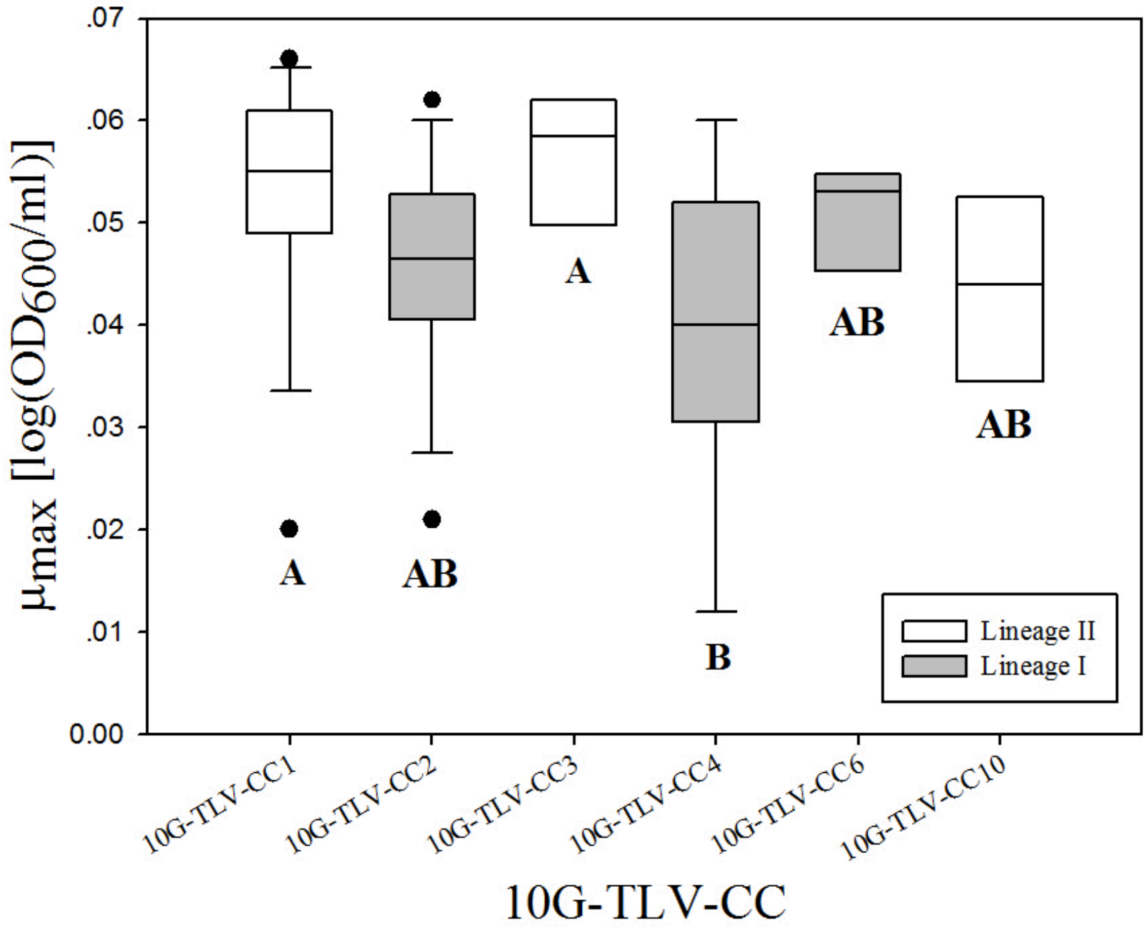

Figure 2.2. Boxplots of the distribution of *L. monocytogenes* growth parameter μ_{max} for six major 10G-TLV-CCs

Boxplots of the distribution of *L. monocytogenes* growth parameter μ_{max} for six major 10G-TLV-CCs (defined as each has five or more isolates) in defined media at 16 °C. 10G-TLV-CC1, 3, and 10 represent lineage II strains, and 10G-TLV-CC2, 4 and 6 represent lineage I strains. The horizontal bar indicates the median for each treatment. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. Filled circles represent values outside the 10th to 90th percentiles. Boxplots with the same letter are not statistically different from each other (overall $\alpha = 0.05$, Tukey HSD correction).

We also assessed whether the source or serotype of isolates may influence μ_{max} in DM. However, a one-way ANOVA model with source effect (human, food, environment and animal as four levels of this effect) or serotype (1/2a, 1/2b and 4b as three levels of this effect) for the growth parameter μ_{max} did not show significant effect ($P > 0.05$) on μ_{max}. Although various studies have characterized *L. monocytogenes* isolates to probe for phenotypic differences between *L. monocytogenes* lineages or serotypes (8, 10, 11, 29, 37), our data suggest that it is necessary to compare between *L. monocytogenes* CCs, which are defined by sequence typing methods such as 10G-MLST, when studying the phenotypes of *L. monocytogenes*, as classification by lineage or serotype may not be able to reflect the genetic basis for phenotype difference between different phenotypes of *L. monocytogenes* clusters.

CONCLUSION

Our findings demonstrated that the 124 *L. monocytogenes* isolates are genetically diverse, and the outbreak-associated isolates were wide-spread through the diversity of *L. monocytogenes*. We also identified that, among the 105 isolates tested, there was considerable variation in maximum growth rate in a nutrient limited growth condition. We found that certain genetic subgroups of lineage II strains could grow faster than some genetic subgroups of lineage I strains under this nutrient limited condition, which may facilitate their persistence in non-host environments, thus leading to a higher risk of food contamination and transmission of this pathogen through foods to humans. Our data highlighted the importance of using molecular typing methods that are amenable to evolutionary analysis for a further understanding of the phylogenetic distribution of *L. monocytogenes* isolates and for the phenotypic characterization of genetic subgroups of *L. monocytogenes*. Classification of *L. monocytogenes* based on DNA

sequence polymorphism, in the long term, may facilitate the surveillance, detection, and control of *L. monocytogenes* strains that are more likely to cause outbreaks and sporadic listeriosis cases.

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CHAPTER 3

EFFICACY OF DIFFERENT ANTIMICROBIALS ON INHIBITION OF *LISTERIA MONOCYTOGENES* GROWTH IN LABORATORY MEDIUM AND ON COLD-SMOKED SALMON

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ABSTRACT

L. monocytogenes is of particular concern in cold-smoked fish products as it can survive curing and cold-smoking, and can subsequently grow from low numbers to potentially hazardous levels during refrigerated storage. The purpose of this study was to (i) quantify the effects of organic acids, nisin, and their combinations on controlling *L. monocytogenes* growth on cold-smoked salmon at refrigeration temperatures, (ii) identify synergistic interactions of binary combinations of these antimicrobials, and (iii) determine if results from laboratory growth media can predict antimicrobial efficacy on cold-smoked salmon. Strains representing the genetic diversity of *L. monocytogenes* lineages I and II were grown in brain heart infusion (BHI) broth as well as on the surface of commercially produced wet-cured, cold-smoked salmon slices at 7^{o}C. BHI broth and cold-smoked salmon were supplemented with sodium diacetate (SDA, 0.14% water phase (w.p.)), potassium lactate (PL, 2% w.p.), nisin (NI, 50 ppm), and binary combinations of inhibitors at the same levels. Cell densities of *L. monocytogenes* were measured over time and used to calculate growth parameters, including initial cell density (N_0), lag phase

(λ), maximum growth rate (μ_{max}), and maximum cell density (N_{max}) for each antimicrobial treatment. N_0 was significantly lowered by addition of NI with a similar average reduction on salmon (2.02 ± 0.99 log(CFU/g)) and in BHI (1.51 ± 0.83 log(CFU/ml)). Among all antimicrobial treatments, the combination of PL and SDA led to the greatest increase in λ both on salmon (7.1 ± 3.6 days) and in BHI (9.7 ± 3.8 days) when compared to the controls. The combination of PL and SDA had synergistic effects on increasing λ and lowering N_{max} both in BHI and on salmon. Among all the treatments tested, the combination of NI and PL led to the greatest reductions in N_{max} on salmon. We observed positive correlations between the growth parameters obtained from BHI broth and cold-smoked salmon, indicating that growth of *L. monocytogenes* in broth, to some extent, qualitatively reflected characteristics of growth on cold-smoked salmon under antimicrobial stresses. Results from BHI could quantitatively predict the variability of growth parameters obtained from salmon for lineage II strains, but not for lineage I strains. Although results from laboratory growth medium may not provide exact predictions of antimicrobial efficacy on cold-smoked salmon, they could be used to rapidly identify effective combinations for further examination on cold-smoked salmon.

INTRODUCTION

Listeria monocytogenes is an opportunistic foodborne pathogen that causes a severe life-threatening disease, particularly in neonates, pregnant women, elderly people and those with a compromised immune system. *L. monocytogenes* can be found in many environments and is capable of surviving under diverse environmental conditions, including those encountered during food processing and refrigerated storage (1). The ability to grow at low temperatures elevates the risk of food contamination and transmission of this pathogen through ready-to-eat (RTE) foods to humans. Characterization of *L. monocytogenes* isolates by multiple subtyping methods (2-4) has shown that strains can belong to one of four distinct genetic lineages (5, 6) with apparent differences in virulence (7, 8), growth under adverse conditions (9, 10) and gene expression related to virulence and stress response (11, 12). While strains in lineage I are widely distributed in foods as well as animals, in many countries this lineage is overrepresented among isolates from sporadic and epidemic cases of human listeriosis, and shows a greater pathogenic potential than lineage II strains (7, 13). Lineage II strains, on the other hand, are most commonly isolated from food and the environment (7, 14), possibly because strains from this lineage can outcompete lineage I strains in some selective media and isolation protocols (15). These genetic variations of *L. monocytogenes* clearly illustrate the importance of using strains representing different lineages in studies on *L. monocytogenes* stress survival and virulence as well as in validation studies for *L. monocytogenes* control strategies.

L. monocytogenes has been identified as of particular concern in RTE smoked fish products including cold-smoked salmon (16-18) because the heat applied during processing is not sufficient to inactivate *L. monocytogenes.* In addition, typical product characteristics of cold-

smoked seafood including pH, water activity, salt, sodium nitrite, and phenolic components are not sufficient to control the growth of *L. monocytogenes* (19, 20). *L. monocytogenes* can multiply from low numbers to potentially hazardous levels in vacuum-packed cold-smoked salmon during long-term refrigerated storage, consequently, this product has been identified as having a high risk per serving for listeriosis (17, 21). During processing and post-smoking handling, cross contamination from the processing plant environment to the product can occur (22, 23). While improved sanitation measures can reduce *L. monocytogenes* contamination (24), complete elimination of *L. monocytogenes* from cold-smoked fish processing environments has been impossible (25). Therefore, it is critical to develop control strategies that prevent or reduce growth of this pathogen in RTE seafood products, including cold-smoked salmon.

One potential method to control *L. monocytogenes* in RTE seafood is the use of bactericidal or bacteriostatic antimicrobial agents, such as nisin, potassium lactate and sodium diacetate. Nisin (NI), a bacteriocin produced by *Lactococcus lactis*, is known to reduce the population of *L. monocytogenes* in various food products, including cold-smoked salmon (26-29). Nisin uses Lipid II as a docking molecule to form pores in the bacterial cell membrane. Lipid II is the membrane-bound peptidoglycan precursor that is essential for bacterial cell-wall biosynthesis. The specific recognition and binding of Lipid II by nisin results in effective permeabilization of the cytoplasmic membrane of vegetative cells and the inhibition of cell wall biosynthesis. This leads to leakage of intracellular fluids, disruption of the proton motive force, and eventually the death of the cell (30, 31). Salts of organic acids, such as potassium lactate (PL) and sodium diacetate (SDA), are widely used in the RTE meat industry as antimicrobial agents (32), and have also been evaluated for use in cold-smoked salmon (21, 33). Organic acids cross the cell membrane in the undissociated form and dissociate in the cytoplasm causing a decrease in

intracellular pH (34), which has significant impacts on cell metabolism, resulting in reduced growth (35, 36).

Combining antimicrobial agents in a food system can often enhance antimicrobial action against pathogenic and/or spoilage organisms (37-39). Studies in laboratory media (40, 41) and studies in food products (21, 33, 42) have been conducted to evaluate the efficacies and identify synergistic effects of antimicrobials. As challenge studies in food matrices can be costly and time-consuming, there is potential to utilize antimicrobial efficacy data generated from studies in laboratory medium to predict efficacy in food matrices. The efficacy of and potential synergy between NI and PL or NI and SDA on *L. monocytogenes* growth in BHI broth or on cold-smoked salmon has rarely been evaluated quantitatively, and potential correlations between growth of *L. monocytogenes* in laboratory medium and in the food matrix treated with antimicrobials are currently unknown. Strong correlations would allow data generated from laboratory medium to be used as an effective and rapid initial screen for antimicrobial efficacy.

Building on our previous study demonstrating the synergistic inhibitory effects of PL and SDA on growth of *L. monocytogenes* in laboratory medium (40), the purpose of this study was to validate NI, PL and SDA, used alone or in binary combinations, as effective antimicrobials against *L. monocytogenes* in modified BHI broth and on vacuum packed cold-smoked salmon at 7^{o}C. Antimicrobials were tested using strains from lineages I and II to assess efficacy across genetic diversity. We were interested in identifying i) significant effects of antimicrobials on *L. monocytogenes* growth parameters in BHI and/or on salmon, ii) significant differences in the effect of antimicrobial on growth parameters dependent on genetic lineage of *L. monocytogenes,*

iii) if synergies existed between antimicrobials when used in combination, and (iv) the correlation between antimicrobial efficacies in BHI broth and on cold-smoked salmon.

MATERIAL AND METHODS

Strains and inoculum preparation. *L. monocytogenes* isolates (Table 3.1) were streaked from frozen stocks of BHI culture stored at -80^{o}C in 15% glycerol onto BHI agar plates and incubated at 37^{o}C for 24 h. Single colonies of each strain were individually inoculated into 5 ml of BHI broth (in 16 mm tubes), and were incubated at 37^{o}C, with shaking (230 rpm) for 18 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 18 h, 50 µl BHI culture was inoculated into 5 ml chemically defined medium (DM) (43) and grown to stationary phase in DM at 16^{o}C statically, as described previously (40).

Table 3.1. Subtype and source information for *Listeria monocytogenes* strains used in this study

Strain	Lineage[a]	Serotype[a]	Ribotype[a]	MLSA[b]	Source[a]	References
FSL J1-194	I	1/2b	1042B	9	Human, sporadic	(44)
FSL F2-693	I	1/2b	1042B	29	Human, sporadic	(45)
FSL L3-051	I	1/2b	1042C	30	RTE salmon	(45)
FSL L4-060	I	1/2b	1043A	1	Smoked fish plant drain	(24)
FSL L4-400	I	1/2b	1052A	1	Smoked fish plant drain	(24)
FSL J1-110	I	4b	1038B	5	Cheese, epidemic	(46)
FSL R2-501	I	4b	1042B	27	Human, epidemic	(46)
FSL J1-126	I	4b	1042B	5	Human, epidemic	(46)
FSL N1-061	I	4b	1044A	7	Salmon brine	(16)
FSL F6-366	I	4b	1044A	6	RTE meat, outbreak	(47)
FSL T1-073	II	1/2a	1023C	46	Raw salmon	(48)
FSL X1-001	II	1/2a	1030A	41	Human, skin lesion	(49)
FSL F2-039	II	1/2a	1030B	39	Human, sporadic	(44)
FSL L4-396	II	1/2a	1039C	31	Smoked fish plant drain	(24)
FSL C1-111	II	1/2a	1039E	48	Human, sporadic	(44)
FSL F2-032	II	1/2a	1045B	45	RTE smoked whitefish	(45)
FSL R2-559	II	1/2a	1053A	51	RTE meat	(47)
FSL L4-151	II	1/2a	1062A	38	Smoked fish plant drain	(24)
FSL F2-237	II	1/2a	1062D	47	RTE smoked salmon	(45)
FSL F2-194	II	N/D	1030A	42	Human, sporadic	(13)

[a]Subtyping and source information can be found in Food Microbe Tracker, available at: http://www.foodmicrobetracker.com

[b]MLSA (Multi-locus Sequence Analysis) data was collected using the methods described by den Bakker et al. (50)

Growth and enumeration of *L. monocytogenes* in BHI broth. BHI broth was modified to have 4.65% waterphase (w.p.) NaCl, pH 6.1 to simulate the levels typically present in commercially processed cold-smoked salmon (40) and supplemented with SDA (Macco Organiques, Inc., Valleyfield, Quebec, Canada), PL (PURASAL Hi Pure P-Plus, PURAC America, Inc.), NI (Nisaplin, contains approximately 2.5% nisin, Danisco, Inc., Denmark) or combinations of two antimicrobials. Growth of *L. monocytogenes* was specifically assessed in BHI broth with seven different treatments including (i) no added inhibitors (CTRL), (ii) 0.14% w.p. SDA, (iii) 2% w.p. PL, (iv) 50 ppm w.p. NI, (v) PLSDA (2% PL + 0.14% SDA), (vi) NISDA (50 ppm NI + 0.14% SDA), or (vii) NIPL (50 ppm NI + 2% PL). As the pK_a values are constants for PL (3.79) and diacetate (4.79, the pK_a for acetate, the base anion of diacetate) (41), the concentrations of undissociated lactate and acetate in a pH 6.1 solution are 0.71 and 0.39 mM/kg for solutions containing PL and/or SDA, respectively. For the remainder of this article, we refer to the water-phase percentages for consistency with recent work evaluating these antimicrobials in food products. For each strain and treatment, 75 ml fresh medium was aseptically aliquoted into sterile, 300-ml Erlenmeyer shake flasks with metal caps (Bellco Glass Co., Vineland, NJ). After chilling to 7^{o}C, the treatment media were inoculated with stationary-phase cells, grown in DM, for a target population of approximately 1 x 10^6 CFU/ml, followed by incubation at 7°C. To monitor *L. monocytogenes* growth, cell density was determined every day, starting from day 0, until growth initiated, and then every other day until entry into stationary phase; measurement was stopped after three time points were taken during stationary phase. For all samples, cultures were diluted with phosphate-buffered saline (PBS) and spiral-plated in duplicate onto both BHI agar and Oxford agar plates (Becton Dickinson, Sparks, MD #222530 and Oxford, Cambridge, UK, SR0140) using an Autoplate 4000 (Spiral Biotech, Inc., Norwood

MA). BHI plates were incubated at 37 ^{o}C for 24 h, and Oxford agar plates were incubated at 30 ^{o}C for 48 h, before colonies were counted with the Colony Counter (Spiral Biotech, Inc.).

Growth and enumeration of *L. monocytogenes* on cold-smoked salmon. Commercially produced wet-cured cold-smoked salmon fillets were stored at -20 ^{o}C and thawed at 4°C overnight. A mixture of natural hardwood and fruitwood was used to cold smoke the salmon. The physicochemical characteristics of the salmon fillets have been described by Kang et al. (51). Salmon slices were weighed (25 ± 0.5g each) and transferred into sterile petri dishes. For each treatment, 700 µl of concentrated PL, SDA, and/or NI solutions were added on the surface of each sample to achieve the same concentration of antimicrobials as used in BHI broth, and spread with sterile plastic cell spreaders. The treated salmon slices were stored for 15 min in a biosafety cabinet to allow the treatment solutions to be absorbed. The treated surface was inoculated with stationary-phase DM cultures diluted in 0.1% sterile peptone water, for a target population of approximately 1 x 10^4 CFU/g. Inoculated salmon slices were then stored in a biosafety cabinet for another 15 min before being transferred into storage bags (oxygen permeability 38.10 cc/m^2 - 40.50 cc/ m^2 at 23°C dry/24 hrs) and packaged using a commercial vacuum sealer (FoodSaver, model V2244). All samples were stored at 7°C. *L. monocytogenes* numbers were enumerated at different time points, based on treatment, including (i) day 0, 1, 2, 7, 12, 17, 23, and 28 for CTRL treatment; (ii) 0, 1, 2, 8, 14, 20, 26, and 32 for SDA treatment; (iii) day 1, 2, 3, 8, 14, 20, 26, and 32 for NI, NISDA and NIPL treatment; and (iv) day 0, 1, 5, 11, 17, 23, 29, 35, and 41 for PL and PLSDA treatment. Vacuum-packed salmon samples were aseptically opened and stomached for 30 s at high speed setting (Seward, Stomacher 400, UK) with 50 to 100 ml of 0.1% sterile peptone water transferred into each bag. Salmon homogenates were spiral-plated on Oxford agar using the Autoplate 4000. Un-inoculated salmon samples were

spiral-plated onto Oxford agar to confirm the absence of *L. monocytogenes*. Oxford agar plates were incubated at 30 ^{o}C for 48 h, before colonies were counted with the Colony Counter (Spiral Biotech, Inc.). The background microbiota (mainly lactic acid bacteria) of the salmon slices used in the present study has been tested (by using nitrite-actidione-polymyxin agar) and discussed by Kang et al (51).

Monitoring of incubation temperatures. Incubator temperature was recorded every 20 min by an automated thermal recorder during the storage of both BHI broth cultures and cold-smoked salmon samples. The average recorded incubation temperature was 7.0 ± 0.5°C.

Growth model and statistical analysis. Measurements of *L. monocytogenes* cell density over time in BHI broth and on cold-smoked salmon were fitted with a three-phase linear model described by Buchanan et al. (52) using the NLStools package (v0.0-11) in R v 2.13.0. Four growth parameters including lag phase (λ, [day]), maximum growth rate (μ_{max}, [log(CFU/ml/)day] for BHI, [log(CFU/g)/day] for salmon), initial cell density (N_0, [log(CFU/ml)] for BHI, [log(CFU/g)] for salmon) and maximum cell density (N_{max}, [log(CFU/ml)] for BHI, [log(CFU/g)] for salmon) were calculated for each strain for each treatment from this model. For each treatment in BHI broth and on salmon, each strain was tested once; multiple strains per lineage were considered replicates.

To better characterize the bactericidal effect of treatments on *L. monocytogenes*, the differences between N_0 of CTRL and each antimicrobial treatment were calculated for each strain as a new growth parameter: N_0 reduction (N_r). The N_{max} reduction (N_{maxr}) was calculated from the difference between N_{max} of CTRL and the antimicrobial treatments for each strain to standardize the effect of antimicrobial treatments on N_{max} reduction. Further, predicted time for 1

log *L. monocytogenes* growth (T_{log}) for each treatment and each strain was calculated from the Buchanan model with the formula $T_{\log} = \lambda + 1/\mu_{max.}$

Two-way analysis of variance (ANOVA) was used to determine if the effect of antimicrobial treatments, genetic lineage, as well as the interaction between treatment and lineage, were significant for each parameter from growth in broth and on salmon, respectively, where the values of the growth parameters served as the response. The linear model used for the ANOVA was:

Y (growth parameter) = treatment | lineage + strain + E(error)

where " | " indicates a full factorial model of the grouped parameters, and strain was treated as a random effect. Significant difference between a given pair of antimicrobial treatments were identified by the Tukey HSD method ($\alpha = 0.05$; JMP 7, SAS Institute, Inc., Cary, NC). Tukey HSD adjusted p-values (adj. P) are reported as significant when < 0.05. To determine if there were significant differences between the responses of lineage I strains and lineage II strains for each antimicrobial treatment, the student's t test was used to compare the values of each growth parameter between lineages within each treatment. To examine the synergistic effects of combinations of the antimicrobials on growth of *L. monocytogenes*, a two-way ANOVA model on the interaction between PL and SDA, NI and SDA, as well as NI and PL, were carried out. In each ANOVA model, the two antimicrobials tested were taken as fixed effects with two levels: presence or absence of the antimicrobial. The linear model used for the ANOVA was:

Y (growth parameter) = A1 | A2 + lineage + strain + E(error)

where " | " indicates a full factorial model of the tested parameters, A1 and A2 indicates a pair of two different antimicrobials among PL, SDA and NI. Lineage was treated as a fixed effect and strain was treated as a random effect. To investigate the correlation between the antimicrobial efficacies in BHI and on salmon, we used linear regression to describe the relationship between growth parameters from salmon and those from BHI. Growth parameters from salmon were fitted with a linear equation as a function of those from BHI using the procedure of linear regression model (JMP 7, SAS Institute, Inc., Cary, NC). The mathematical equation of the fitting is:

$$\text{Growth Parameter (salmon)} = \alpha + \beta \times \text{Growth Parameter (BHI)}.$$

where α is the intercept, and β is the estimated coefficient (slope). As the correlations varied among lineages and treatments with or without NI, we split the data into two groups, one with parameters from treatments with organic acid salts only (PL, SDA, PLSDA), the other with parameters from treatments with NI (NI, NISDA, NIPL); and we analyzed the data by lineage within each group. Since organic acid salts did not show any effect on N_r, this parameter was not included in the analysis for organic acid treatments; the growth parameters λ, μ_{max}, T_{log}, N_{max} and N_{maxr} were examined. For treatments with NI, we focused on the growth parameters N_r, N_{max} and N_{maxr}, which described the bactericidal effect of NI.

RESULTS

We quantified the effects of antimicrobials on growth of 20 *L. monocytogenes* strains representing diverse genetic backgrounds in BHI broth and on cold-smoked salmon (Table 3.1) Strains FSL T1-073 and FSL X1-001 did not show obvious growth for most of the treatments on

salmon. Hence, the analyses reported here were based on 20 strains for BHI (10 strains in lineage I and 10 strains in lineage II) and 18 strains for salmon (10 strains in lineage I and 8 strains in lineage II).

Treatments with NI significantly reduced initial cell density. Among the six antimicrobial treatments, ANOVA indicated that the growth parameter N_r for SDA, PL, and PLSDA was not significantly different from zero (adj. $P > 0.05$); while all treatments containing NI had N_r that were significantly greater than zero across *L. monocytogenes* genetic lineages (adj. $P < 0.05$), both in BHI and on salmon (Table 3.2; Fig. 3.1A, B). N_r caused by treatments with NI (alone or in combination) ranged from 0.34 to 3.82 log(CFU/ml) in BHI and from 0.08 to 4.00 log(CFU/g) on salmon. In BHI, a comparison of N_r between lineages within each treatment using student's t test revealed that N_r of lineage II strains were significantly greater in the presence of NI, as compared to lineage I strains (Table 3.2; Fig. 3.1A; student's t test: $P < 0.05$). As shown in Table 3.2 and Fig. 3.1A, the average N_r of lineage II strains in BHI for NI, NISDA and NIPL were all more than half log(CFU/ml) higher than those of lineage I strains. On salmon, a lineage-dependent effect of NI on N_r was observed (Table 3.2; student's t test: $P < 0.05$), opposite of that in BHI, where average N_r of lineage I strains was more than half log(CFU/ml) greater than that of lineage II strains. In BHI, NI and PL in combination increased N_r compared to using NI alone or NI combined with SDA (adj. $P < 0.05$). However, the average N_r of NIPL was not significantly different from that of NI on salmon (adj. $P > 0.05$). Adding SDA to NI led to an antagonistic effect on N_r compared to using NI alone on salmon, which was identified by a significant interaction effect ($P < 0.05$) for a two-way ANOVA between treatments with NI and treatments with SDA. Taken together, these data indicate that addition of SDA or PL to NI did not significantly increase the bactericidal efficacy of NI on salmon.

Table 3.2. Antimicrobial treatment effects and lineage-specific effects on maximum growth rate and initial cell density reduction for *Listeria monocytogenes* at 7°C

Treatment	BHI					Salmon				
	Total		Lineage I		Lineage II	Total		Lineage I		Lineage II
N_r: Broth [log(CFU/ml)]; Salmon [log(CFU/g)]										
SDA	0.02 ± 0.09[a]	A[b]	-0.02 ± 0.09[c]	=[d]	0.06 ± 0.09	0.01 ± 0.08	A	0.01 ± 0.10	=	0.01 ± 0.05
PL	0.10 ± 0.15	A	0.08 ± 0.13	=	0.12 ± 0.17	0.04 ± 0.08	A	0.05 ± 0.09	=	0.02 ± 0.07
PLSDA	0.15 ± 0.20	A	0.15 ± 0.21	=	0.16 ± 0.22	0.01 ± 0.09	A	0.00 ± 0.12	=	0.03 ± 0.05
NI	1.51 ± 0.83	B	1.20 ± 0.70	<	1.82 ± 0.90	2.02 ± 0.99	C	2.30 ± 1.14	>	1.67 ± 0.68
NISDA	1.43 ± 0.78	B	1.17 ± 0.78	<	1.69 ± 0.76	1.45 ± 0.79	B	1.51 ± 0.89	=	1.38 ± 0.69
NIPL	2.01 ± 0.95	C	1.69 ± 0.99	<	2.32 ± 0.90	2.22 ± 0.82	C	2.40 ± 1.08	=	1.99 ± 0.23
μ_{max}: Broth [log(CFU/ml)/day]; Salmon [log(CFU/g)/day]										
CTRL	0.38 ± 0.07	A	0.36 ± 0.05	=	0.39 ± 0.09	0.32 ± 0.08	AB	0.33 ± 0.06	=	0.30 ± 0.10
SDA	0.33 ± 0.05	ABC	0.30 ± 0.03	=	0.36 ± 0.06	0.23 ± 0.07	BCD	0.24 ± 0.06	=	0.22 ± 0.08
PL	0.28 ± 0.07	CD	0.27 ± 0.09	=	0.29 ± 0.06	0.15 ± 0.06	CD	0.14 ± 0.07	=	0.16 ± 0.04
PLSDA	0.17 ± 0.11	E	0.18 ± 0.14	=	0.16 ± 0.09	0.09 ± 0.04	D	0.10 ± 0.02	=	0.09 ± 0.06
NI	0.37 ± 0.05	AB	0.34 ± 0.05	=	0.39 ± 0.04	0.47 ± 0.31	A	0.59 ± 0.36	>	0.33 ± 0.13
NISDA	0.31 ± 0.06	BCD	0.27 ± 0.06	<	0.34 ± 0.05	0.32 ± 0.21	AB	0.39 ± 0.24	>	0.22 ± 0.10
NIPL	0.27 ± 0.03	D	0.27 ± 0.02	=	0.27 ± 0.04	0.32 ± 0.23	ABC	0.44 ± 0.24	>	0.17 ± 0.04

[a]Results are summarized by means ± standard deviations for 20 strains for BHI (10 strains in lineage I and 10 strains in lineage II) and 18 strains for salmon (10 strains in lineage I and 8 strains in lineage II), each strain was tested once (n = 20 for BHI per treatment, n = 18 for salmon per treatment).

[b]Means within a given column with the same letter are not statistically different from each other (overall $\alpha = 0.05$, Tukey's correction**).**

[c]Data are separated into lineage I and II for each treatment due to the significant lineage-dependent effect on initial cell density reduction and on maximum cell density both in BHI broth and on cold smoked salmon according to student's t test results.

[d]Comparison results between lineage I and II for CTRL, treatments with organic acid salts and treatments with nisin from student's t test. An equal symbol indicates no significant difference between the two lineages for the treatment, while a < or > indicates that lineage I had a significantly lower, or significantly higher, average value for the treatment compared to lineage II.

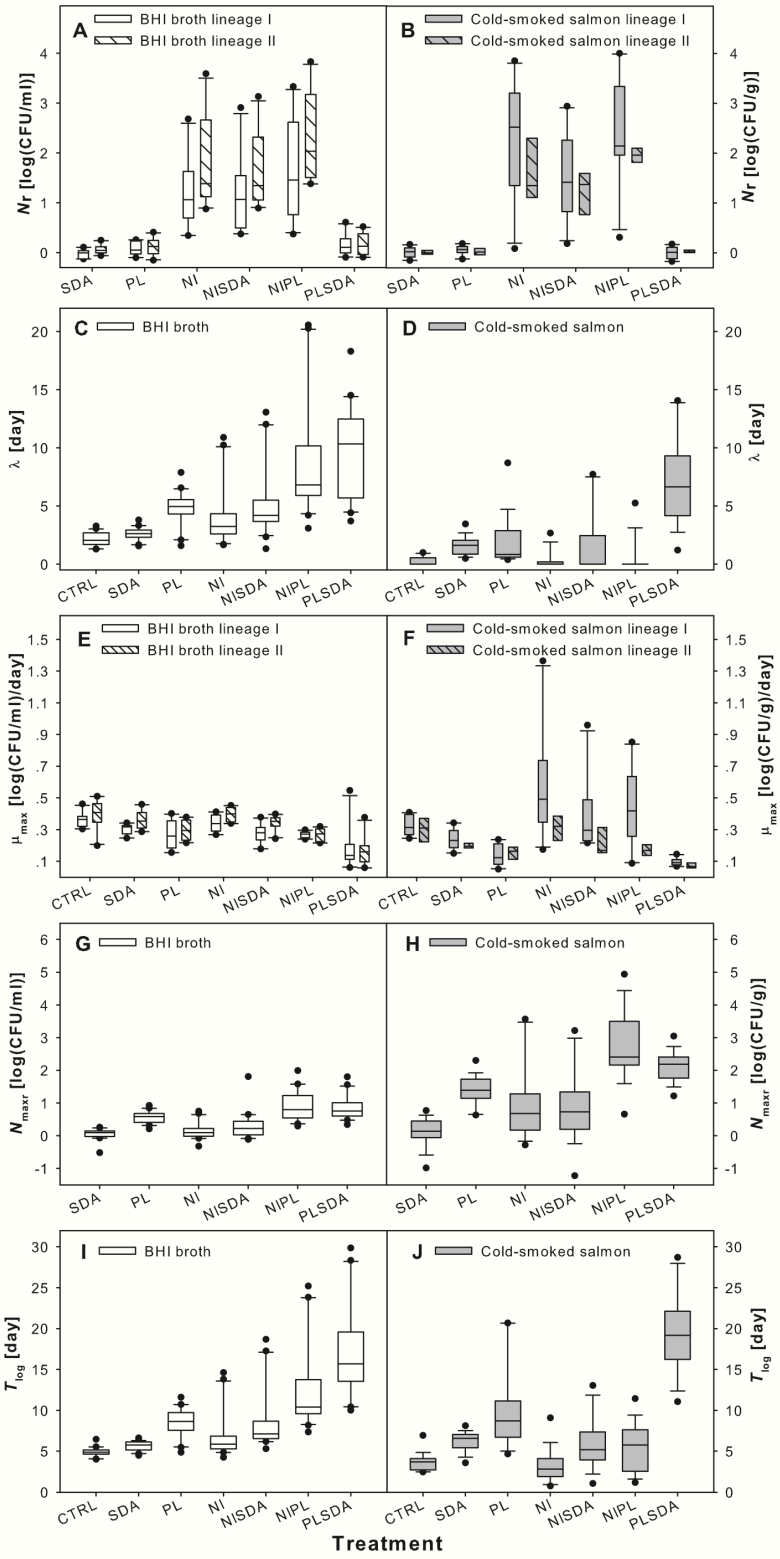

Figure 3.1. Boxplots of the distribution of *L. monocytogenes* growth parameters for each growth inhibitor treatment in modified brain heart infusion broth and on cold smoked salmon at 7°C

(A, B) Initial cell density reduction (C, D) lag phase, (E, F) maximum growth rate, (G, H) maximum cell density reduction, and (I, J) time for growth of 1 log(CFU/ml) for BHI broth, 1 log(CUF/g) for cold smoked salmon, from initial cell density. Data are separated into lineage I and II for A, B, E and F due to the significant lineage-dependent effect on initial cell density reduction and maximum growth rate for treatments with NI. The horizontal bar indicates the median for each treatment. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. Filled circles represent values outside the 10th to 90th percentiles. Results are from 20 strains for BHI (10 strains in lineage I and 10 strains in lineage II) and 18 strains for salmon (10 strains in lineage I and 8 strains in lineage II), each strain was tested once (n = 20 for BHI per treatment, n = 18 for salmon per treatment).

The combination of PL and SDA significantly extended lag phase. Among the six antimicrobial treatments, NISDA, NIPL and PLSDA showed significantly longer λ compared to CTRL in BHI (Table 3.3; Fig. 3.1C; adj. $P < 0.05$), but only PLSDA was observed to significantly extend λ of *L. monocytogenes* on salmon (Table 3.3; Fig. 3.1D; adj. $P < 0.05$). Beyond the comparisons of growth parameter means from each treatment, ANOVA results showed a significant interaction of PL and SDA on extending λ in BHI ($P < 0.0001$) and on salmon ($P < 0.001$), indicating a synergistic effect. SDA or PL alone had little effect on λ (adj. $P > 0.05$), but addition of SDA to PL significantly increased average λ to 10 days in BHI and 7 days on salmon as compared to CTRL (Table 3.3; adj. $P < 0.05$). Due to rapid onset of *L. monocytogenes* growth on cold-smoked salmon treated with NI, we were unable to calculate λ for some of the NI, NISDA and NIPL treatments, and defined those as λ = 0 day. For treatments with NI, average lag times were much shorter in salmon than in BHI (Table 3.3; Fig. 3.1C, D). Additionally, an antagonistic effect on λ of NI and PL was observed for growth on salmon, identified by a significant interaction effect ($P < 0.05$) for a two-way ANOVA between treatments with PL and treatments with NI. NI alone did not have any effect on λ, but adding NI to PL antagonistically shortened the average λ of PL by more than 1 day ($P < 0.01$). This effect was not observed in BHI.

Table 3.3. Antimicrobial treatment effects on lag phase, maximum cell density reduction, and predicted time for growth of one log for *Listeria monocytogenes* at 7°C

Treatment	λ[a]				N_{maxr}				T_{log}			
	Broth (days)		Salmon (days)		Broth [log(CFU/ml)]		Salmon [log(CFU/g)]		Broth (days)		Salmon (days)	
CTRL	2.2 ± 0.6	A[b]	0.2 ± 0.4	A	0.00 ± 0.00	A	0.00 ± 0.00	A	4.9 ± 0.5	A	3.6 ± 1.1	A
SDA	2.6 ± 0.5	AB	1.6 ± 0.8	A	0.05 ± 0.16	AB	0.12 ± 0.42	A	5.7 ± 0.6	AB	6.2 ± 1.2	A
PL	4.7 ± 1.5	AB	1.9 ± 2.1	A	0.57 ± 0.19	C	1.41 ± 0.44	BC	8.5 ± 1.72	B	9.9 ± 4.7	B
NI	4.1 ± 2.6	AB	0.4 ± 0.8	A	0.13 ± 0.24	AB	1.00 ± 1.13	B	6.9 ± 2.9	AB	3.2 ± 2.0	A
NISDA	5.3 ± 3.1	B	1.6 ± 2.5	A	0.30 ± 0.41	B	0.92 ± 1.08	B	8.7 ± 3.8	B	5.8 ± 3.2	A
NIPL	9.2 ± 5.3	C	0.6 ± 1.4	A	0.91 ± 0.44	D	2.75 ± 1.04	D	13.0 ± 5.5	C	5.4 ± 2.8	A
PLSDA	9.7 ± 3.8	C	7.1 ± 3.6	B	0.86 ± 0.35	D	2.10 ± 0.45	C	17.4 ± 5.9	D	19.5 ± 5.0	C

[a]Results are summarized by means ± standard deviations for 20 strains for BHI (10 strains in lineage I and 10 strains in lineage II) and 18 strains for salmon (10 strains in lineage I and 8 strains in lineage II), each strain was tested once (n=20 for BHI per treatment, n=18 for salmon per treatment).
[b]Means within a given column with the same letter are not statistically different from each other (overall $\alpha = 0.05$, Tukey's correction**).**

PL and PLSDA significantly lowered the maximum growth rate of *L. monocytogenes.*

Average μ_{max} across *L. monocytogenes* genetic lineages was significantly lowered by PL PLSDA, NISDA and NIPL in BHI, but only by PL and PLSDA on salmon (Table 3.2; Fig. 3.1E, F; adj. P <0.05). ANOVA models with lineage effect for the growth parameter μ_{max} showed significant differences in μ_{max} across all treatments dependent on lineage for BHI ($P < 0.05$) and salmon ($P < 0.01$). At the same time, analysis using student's t test comparing μ_{max} of lineages within each treatment revealed that μ_{max} of lineage II strains was significantly greater in BHI treated with NISDA (student's t test: $P < 0.05$), but significantly lower on salmon treated with NI, NISDA and NIPL (student's t test: $P < 0.05$), as compared to lineage I strains (Table 3.2; Fig. 3.1E, F). In contrast, there was no difference between lineages for treatments containing only organic acid salts in BHI or on salmon (Table 3.2; Fig. 3.1E, F). In addition to the lineage-specific effects on μ_{max} for treatments containing NI, significantly greater variance of μ_{max} in the presence of NI (alone and in combination) was observed on salmon as compared to BHI (Bartlett's test, $P < 0.0001$ (NI), $P < 0.0001$ (NISDA) and $P < 0.0001$ (NIPL)). SDA alone did not have a significant effect on μ_{max}, but when combined with PL, the combination had an additive effect that led to a significantly lower average μ_{max} in BHI, as compared to using PL or SDA alone (Table 3.2; adj. $P < 0.05$). While the average μ_{max} of PLSDA was significantly lower than CTRL on salmon (adj. $P < 0.05$), the binary combination was not more effective than using PL or SDA alone in reducing the average μ_{max} (adj. $P > 0.05$). This could be due to the large variation of μmax values for those two treatments, especially for lineage I strains on salmon (Fig. 3.1F).3.4. Treatments with NI and PL significantly decreased maximum cell density

PL, NISDA, NIPL and PLSDA lowered N_{max} in BHI and on salmon, while NI lowered N_{max} on salmon only (Table 3.3; Fig. 3.1G, H; adj. $P < 0.05$). As shown in Table 3.3, NIPL and

PLSDA had the greatest N_{maxr} in BHI, with a maximum N_{maxr} exceeding 1 log(CFU/ml); while only NIPL had the greatest N_{maxr} on salmon, with a maximum N_{maxr} exceeding 3 log(CFU/g). In BHI, SDA or NI alone did not have any effect on N_{maxr}. Combining SDA with NI achieved a average N_{maxr} 1.41 log(CFU/g) on salmon which was significantly greater than zero (adj. $P < 0.05$) but was not significantly different from using SDA or NI alone (Table 3.3; Fig. 3.1G; adj. $P > 0.05$). SDA synergistically enhanced the effect of PL on increasing N_{maxr} both in BHI ($P < 0.05$) and on salmon ($P < 0.0001$), which was identified by a significant interaction effect for a two-way ANOVA between treatments with PL and treatments with SDA. NI and PL had an additive effect on salmon, where combining NI and PL caused a significantly higher N_{maxr}, as compared to using NI or PL alone (Table 3.3, Fig. 3.1H; adj. $P < 0.05$). Significantly greater variance of N_{maxr} was observed on salmon (Fig. 3.1H) as compared to BHI (Fig. 3.1G) for most of the antimicrobial treatments (Bartlett's test: $P < 0.001$ (SDA), $P < 0.001$ (PL), $P < 0.0001$ (NI), $P < 0.001$ (NISDA) and $P < 0.001$ (NIPL), except PLSDA ($P > 0.05$)). This suggests a greater variation of the final cell density among different strains under antimicrobial treatment stresses for *L. monocytogenes* on salmon, as compared to BHI.

PL and PLSDA significantly extended predicted time for growth of 1 log while NI antagonistically shortens predicted time for growth of 1 log when added to PL. The calculated T_{log} parameter combines the influence of λ and μ_{max} on early exponential phase growth, and is thus a better indicator of the antimicrobial efficacy of the treatments. In BHI, PL, NISDA, NIPL and PLSDA all significantly extended T_{log} as compared to CTRL (Table 3.3; Fig. 3.1I; adj. $P < 0.05$); while on salmon, only PL and PLSDA significantly extended T_{log} (Table 3.3; Fig. 3.1J; adj. $P < 0.05$). PLSDA was most effective in preventing *L. monocytogenes* to achieve growth of 1 log with an average T_{log} exceeding 17 days in BHI and 19 days on salmon. ANOVA results

indicate that SDA or NI alone did not have an effect on T_{log}. However, adding SDA to PL synergistically extended average T_{log} of PL by about 9 days both in BHI ($P < 0.0001$) and on salmon ($P < 0.001$), as compared to using PL alone. On the other hand, addition of NI to PL showed an antagonistic effect on T_{log} on salmon ($P < 0.01$), shortening average T_{log} from 10 days to 5 days as compared to using PL alone. Although the combination of NI and SDA significantly extended average T_{log} in BHI to about 9 days compared to CTRL (adj. $P < 0.05$), it did not lead to significant difference from using SDA or NI alone or show any effect on T_{log} on salmon (adj. $P > 0.05$).

Correlation of growth parameters from BHI broth to those from cold smoked salmon were lineage-dependent. Multivariate analysis showed positive correlations between a growth parameter from BHI and the same parameter from salmon, indicating that the growth of *L. monocytogenes* in BHI, to some extent, reflected the characteristics of growth on salmon under antimicrobial stress. As the correlations varied among lineages and treatments with or without nisin, we split the data into two groups, one with parameters from treatments with organic acid salts only (SDA, PL, PLSDA), the other with parameters from treatments with NI (NI, NISDA, NIPL), and analyzed the data by lineage within each group. Coefficients (intercept and slope) and coefficients of determination (R^2) for the fitted linear equations describing a growth parameter from salmon as a function of its corresponding parameter from BHI are presented in Table 3.4. R^2 for parameters for lineage II strains were higher than those for lineage I strains. This indicates that those growth parameters from BHI had a relatively stronger correlation with those from salmon for lineage II strains.

Table 3.4. Coefficients and coefficients of determination of linear regression for *L. monocytogenes* growth parameters in BHI and on cold-smoked salmon

Growth parameter[a]	R^2	Intercept (α)			Slope (β)		
		Estimate	Std Error	P	Estimate	Std Error	P
Treatments with Organic acid salt (SDA, PL, PLSDA)							
Lineage I							
λ	0.26	0.53	1.24	0.6719	0.64	0.20	**0.0038**
μ_{max}	0.11	0.10	0.04	**0.0154**[b]	0.25	0.14	0.0779
T_{log}	0.37	4.05	2.19	0.0752	0.78	0.19	**0.0003**
N_{max}	0.33	-3.49	2.73	0.2115	1.21	0.33	**0.001**
N_{maxr}	0.25	0.82	0.22	**0.001**	1.05	0.35	**0.0052**
Lineage II							
λ	0.58	0.62	0.57	0.2845	0.42	0.08	**<0.0001**
μ_{max}	0.26	0.06	0.04	0.1557	0.37	0.13	**0.0109**
T_{log}	0.61	3.74	1.63	**0.0319**	0.72	0.12	**<0.0001**
N_{max}	0.75	-8.67	1.87	**0.0001**	1.80	0.22	**<0.0001**
N_{maxr}	0.66	0.11	0.19	0.5769	1.83	0.28	**<0.0001**
Treatments with nisin (NI, NISDA, NIPL)							
Lineage I							
N_r	0.03	1.80	0.38	**<0.0001**	0.24	0.42	0.3459
N_{max}	0.06	1.26	3.75	0.7383	0.60	0.45	0.1908
N_{maxr}	0.27	1.03	0.27	**0.0007**	1.36	0.42	**0.0033**
Lineage II							
N_r	0.51	0.80	0.20	**0.0008**	0.50	0.10	**<0.0001**
N_{max}	0.64	-9.77	2.55	**<0.0009**	1.87	0.30	**<.00001**
N_{maxr}	0.58	0.55	0.26	**0.0458**	2.03	0.37	**<0.0001**

[a]Growth parameters from salmon were fitted with a linear equation as a function of those from BHI for treatment with organic acid salts only (PL, SDA, PLSDA) and treatment with nisin (NI, NISDA, NIPL) per lineage.

[b] P values showing significant effects were bolded, α=0.05

Overall, across antimicrobial treatments, we found that the relatively high R^2 values (Table 3.4) for lineage II strains make it possible to predict the variability of these growth parameters from salmon by data from BHI, while the variability of growth parameters of lineage I strains were relatively unpredictable. For treatments with organic acid salts, R^2 for lineage I strains indicates that only 33% of the variance in N_{max} from salmon was accounted for by change in N_{max} from BHI compared to 75% for lineage II (Table 3.4), where salmon and BHI parameter pairs for lineage I strains were distributed much more randomly compared to lineage II strains (Fig. 3.2). The bacteriostatic effects of organic acids were more pronounced in BHI than on salmon for lineage II strains. The slope parameter in the regression showed that λ from salmon was a factor of 0.42 of that from BHI for lineage II strains treated with organic acid salts ($P < 0.0001$), the intercept was not significantly different from zero ($P > 0.05$). This suggests that the organic acid treatments in BHI are approximately 2.38 times as effective as on salmon in extending λ ($R^2 = 0.58$). Both the intercept ($P < 0.05$) and slope ($P < 0.0001$) of T_{log} were significantly different from zero (Table 3.4), which means we can predict 61% ($R^2 = 0.61$) of the variability of T_{log} of salmon by T_{log} of BHI using the equation $T_{log}(\text{salmon}) = 3.74 + 0.72 \times T_{log}(\text{BHI})$. N_{maxr} from salmon was a factor of 1.83 of it from BHI ($P < 0.0001$), the intercept of it was not significantly different from zero ($P > 0.05$), indicating that the organic acid treatments on salmon are estimated to be 1.83 times as effective in BHI in reducing the N_{max} ($R^2 = 0.66$). As the R^2 for μ_{max} was very low for both lineages, μ_{max} was relatively unpredictable for both lineages (Table 3.4).

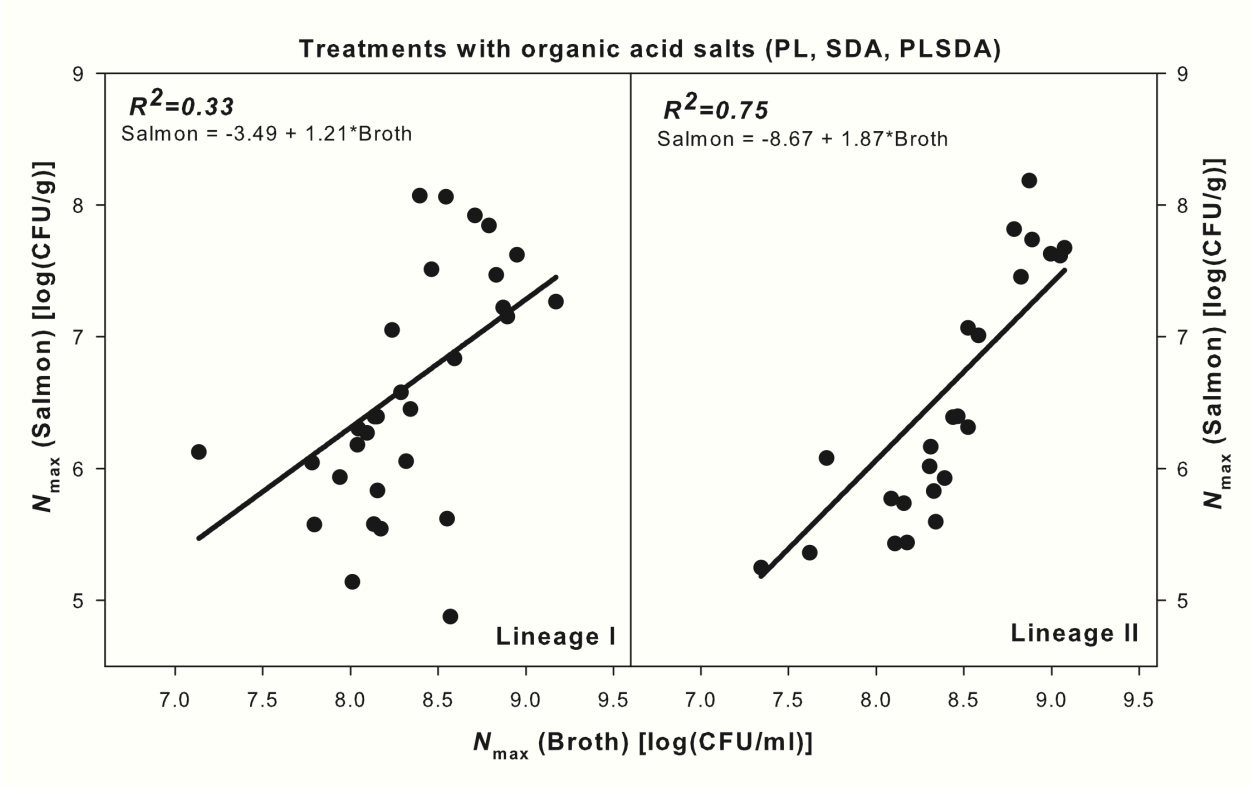

Figure 3.2. Linear fit of N_{max} values from cold smoked salmon by N_{max} values from modified brain heart infusion broth for treatments with organic acid salts

Data for lineage I and II strains are presented separately due to significant differences in correlations dependent on lineage.

For antimicrobial treatments containing NI, both the intercept and slope for N_r and N_{maxr} were significantly different from zero (Table 3.4), and the R^2 was 0.51 and 0.58 (Table 3.4), respectively. This means that we can predict 51% of the N_r and 58% of the N_{maxr} of salmon by those parameters of BHI using the equations $N_r(\text{salmon}) = 0.80 + 0.50 \times N_r(\text{BHI})$ and $N_{maxr}(\text{salmon}) = 0.55 + 2.03 \times N_{maxr}(\text{BHI})$. The equation of N_{maxr} shows that, the N_{maxr} of salmon was at least 0.55 log(CFU/g) higher that it of BHI, indicating a better effect of treatments with NI on increasing N_{maxr}.

Although the positive correlation coefficients from multivariate analysis and the positive slope parameters from the linear regression both indicate similarities between *L. monocytogenes* growth characteristics under antimicrobial stress in BHI and on salmon, quantitative prediction of the performance of antimicrobials on salmon was only possible for lineage II strains, but not for lineage I strains of *L. monocytogenes*.

DISCUSSION

We investigated the effects of the antimicrobials SDA, PL, NI, singly and in combination, on the growth of strains representing two evolutionary lineages of *L. monocytogenes* in modified BHI broth and on cold-smoked salmon at 7°C. Statistical analysis of the growth parameters λ, μ_{max}, N_0, N_{max}, N_r, N_{maxr} and T_{log} showed that most of the binary combinations of antimicrobials had greater effects on inhibiting growth of *L. monocytogenes*, as compared to use of a single antimicrobial alone. Although treatments with NI effectively reduced the initial contamination level of *L. monocytogenes*, the combination of PL and SDA was most effective on inhibiting growth of *L. monocytogenes* during the length of refrigerated storage. Among the binary combinations, some exhibited synergistic effects, indicating that the antimicrobial effect of the

combination is greater than the sum of the effects of the individual antimicrobials, such as the synergistic effects identified between PL and SDA on extending λ and T_{log}. Significant lineage-dependent effects were identified for some antimicrobial treatments. NI had lineage-dependent effects on μ_{ma} as well as on N_r. Correlations between growth parameters in BHI and on salmon revealed that the variability of bactericidal effects of treatments with NI and bacteriostatic effects of treatments with organic acid salts on salmon could be partly predicted by data from BHI broth only for *Listeria monocytogenes* lineage II strains, but not for lineage I strains.

Bactericidal effects of NI on *L. monocytogenes* are highly strain dependent. Our data showed that treatment with NI alone or combined with PL or SDA caused significant, but highly variable, reduction of initial cell populations for *L. monocytogenes* strains in BHI and on salmon at 7°C. The high variation in N_r indicates that the response of an individual strain of *L. monocytogenes* might lead to an inappropriate estimate of efficacy of NI on cold-smoked salmon, highlighting the importance of genetic diversity in validating antimicrobials in a food system. While multi-strain cocktails of *L. monocytogenes* strains have been used in previous studies on the bactericidal efficacy of NI in food matrices (29, 53, 54), we individually investigated 18 distinct *L. monocytogenes* isolates from a wide range of sources and diverse genetic backgrounds for their susceptibility against NI applied to cold-smoked salmon. The difference between the NI sensitivities of any two *L. monocytogenes* strains tested was up to 4 log(CFU/g). A large variation in sensitivity to NI was also observed by Rasch and Knøchel (55), who examined a collection of 381 *L. monocytogenes* strains on Tryptic soy agar containing NI, although the concentrations of NI used were, in general, lower than those used here. For example, they found two strains that were able to grow weakly on 500 IU/ml (12.5 ppm), and while 1% of strains could not grow at 10 IU/ml (0.25 ppm), the majority of strains were able to grow at 100 IU/ml

(2.5 ppm). Ukuku and Shelef (56) also demonstrated that sensitivity of *L. monocytogenes* to NI at pH 5.0, 6.0 and 7.0 was strain dependent.

PLSDA inhibits *L. monocytogenes* growth during extended refrigerated storage. Based on comparisons of λ and T_{log} among different treatments, PLSDA was the most effective for controlling *L. monocytogenes* during extended refrigerated storage. PLSDA completely inhibited *L. monocytogenes* growth for up to 2 weeks on cold-smoked salmon and 1 log growth was achieved after 11 ~ 29 days. Synergistic effects of PL and SDA on extending λ, T_{log} and lowering N_{max} of *L. monocytogenes* were observed both on cold-smoked salmon and in BHI broth. These results are in agreement with previous studies demonstrating the bacteriostatic effect of organic acids against *L. monocytogenes* and the synergistic effect between potassium lactate and sodium diacetate in controlling *L. monocytogenes* on RTE foods, including frankfurters (57, 58), turkey products (39), sausages (37, 59), ham (60) and cold-smoked salmon (21, 33). As the contamination level of *L. monocytogenes* on cold-smoked salmon is normally low (61), and controlling the growth of *L. monocytogenes* is important in lowering the risk of infection, addition of PLSDA during processing could be an effective control strategy against *L. monocytogenes* on cold-smoked salmon.

Addition of organic acids does not increase the bactericidal effect of nisin. NIPL and NISDA had similar effects on N_r of *L. monocytogenes* as NI alone on salmon, indicating that the bactericidal effect of NI was not enhanced by addition of an organic acid. Similar results have been reported for the combination of NI and organic acids (or their salts) on reducing initial densities of *L. monocytogenes* on products such as turkey frankfurters (62), sausages, ham (63-65), and cold-smoked fish products (29). In these reports, addition of organic acid did not

significantly increase the initial reduction in cell density achieved by NI alone. Some studies have demonstrated that NI can significantly increase the length of lag phase and reduce the growth rate of *L. monocytogenes* on cold-smoked fish products (42) and ham (66) when used in conjunction with lactate, compared to NI or lactate alone. In many cases, the combination of NI and an organic acid lead to overall lower *L. monocytogenes* numbers after the duration of refrigerated storage compared to use of organic acid alone, as demonstrated on ham and sausage (63-65). Here we show that NI works additively with PL in lowering N_{max}. While the maximum cell density was reduced by the addition of PL to NI, the predicted time for *L. monocytogenes* to grow 1 log on cold smoked salmon was significantly shorter for the combination of NI and PL compared to the combination of PL and SDA, indicating that the combination of organic acids and nisin may not be as effective at controlling growth of this pathogen as combinations of organic acids alone. In summary, the addition of organic acids did not enhance the bactericidal effect of NI, and NI had negative effects on the bacteriostatic effects of the organic acid salt PL.

The bactericidal effect of NI is lineage-dependent. Lineage-dependent effects were observed for antimicrobial treatments with NI in BHI, where lineage I strains were more resistant to NI than lineage II strains. In addition, after the initial decrease in cell density due to NI exposure, lineage I strains tended to grow faster than lineage II strains on salmon treated with NI. Since this difference in growth was not observed in BHI for all the treatments with NI, growth data from BHI broth could not fully predict the lineage-dependent inhibitory effect of NI on *L. monocytogenes* strains on salmon. While the effects of *L. monocytogenes* lineage on differences in nisin resistance have not been assessed previously, variation among lineages for other stress resistance phenotypes has been reported. For example, lineage II strains were more sensitive to gastric stress in a dynamic gastric system compared to lineage I strains (67), and lineage I and III

strains were more resistant to salt stress compared to lineage II strains (68). The presence of lineage-dependent differences in stress resistance phenotypes also depends on the type of stress *L. monocytogenes* experiences. For example, lineage I and lineage II strains had similar D-values when exposed to heat stress at 55°C (9). In this study, we found that the effect of genetic lineage was minor on low temperature growth of *L. monocytogenes* in the presence of organic acids, both on cold-smoked salmon and in BHI broth. This result is in agreement with Stasiewicz et al. (40) who found no significantly differences for growth parameters (λ, μ_{max}, N_0, N_{max}) between *L. monocytogenes* lineage I and II when antilisterial efficacies of organic acids were tested in BHI broth at 7^{o}C. Taken together, these studies highlight that lineage-dependent differences in *L. monocytogenes* stress resistance can be significant for many types of stresses, including NI stress. It is necessary to include the effect of lineage into analyses of antimicrobial efficacies against *L. monocytogenes.*

Correlations of antimicrobial effects on *L. monocytogenes* growth inhibition in BHI broth and on cold smoked salmon are lineage-dependent. We identified overall positive correlations between *L. monocytogenes* growth parameters in modified BHI broth and on cold-smoked salmon. In general, all of the slope parameters from the fitted linear equation for each growth parameter were positive, indicating that growth of *L. monocytogenes* in BHI, to some extent, reflects the characteristics of growth on salmon under antimicrobial stress. This suggests that BHI broth with the same pH and salt concentration as cold smoked salmon can be used to rapidly identify effective single antimicrobial or combinations for further examination on cold-smoked salmon. However, quantitative prediction of the performance of antimicrobials on salmon was only possible for lineage II strains rather than lineage I strains of *L. monocytogenes*. The unpredictability of the antimicrobial efficacies for lineage I strains make it difficult to use

data from BHI broth to accurately predict the variability of the antimicrobial efficacies against overall *L. monocytogenes* strains on cold smoked salmon.

For many growth parameters, the variation in parameters from BHI broth was much lower than the variation in parameters from cold smoked salmon, especially for lineage I strains. In most cases, antimicrobials appeared to have a greater effect on growth inhibition in BHI compared to on salmon. For example, for lineage II strains, the approximately 1:2 ratio in λ from salmon to that from BHI indicates that organic acid treatments in BHI were approximately twice as effective as on salmon in extending λ of *L. monocytogenes*. Similarly, Vogel et al. (33) reported a more pronounced antilisterial effect of identical concentrations of PL (2%) and SDA (0.14%) in salmon homogenate and salmon juice. They found that PLSDA completely inhibited the growth of *L. monocytogenes* for over 28 days in cold-smoked salmon juice and 27 days in cold-smoked salmon homogenate, compared to the results from the present study (up to 18 days in BHI, 14 days on salmon). We directly applied the antimicrobials on the surface of cold-smoked salmon slices, in comparison to this, Vogel et al. (33) added antimicrobials into salmon homogenate or juice. We speculate that a more homogenous distribution of antimicrobials, when used in liquid based evaluation systems, such as the BHI broth used in this study, and the salmon juice used by Vogel et al., leads to a broader and longer interaction between antimicrobials and target cells, which could reduce variability in the bacterial response to the antimicrobial.

In addition to the distribution of antimicrobials, other physicochemical factors of the food matrix could contribute to increased variability in antimicrobial efficacy. For example, Jung et al. (69) reported a decrease in NI efficacy in food containing fat . It was suggested that fat could exert a negative impact on NI activity by binding NI via hydrophobic sites thereby making it

unavailable for bacterial inhibition (69, 70). Our data suggest that the fat content of salmon does not affect the efficacy of nisin, as the salmon used had a high content of lipids, ranging from 7.4 - 10.4%, and we did not observe a decrease in NI efficacy on salmon compared to in BHI. However, as phenolic compounds are important for the preservation properties of smoked products (71), one may speculate that the phenolic compounds obtained from smoking process (about 184.21 mg GAE/100g for the salmon in the present study; (51)) might have interacted with NI, exerting a better inhibitory effect against *L. monocytogenes* and overcoming the negative impact of salmon fat. It is also important to note that the complex nature of phenolic compounds in the cold-smoked salmon could be influenced by the type of wood used for smoking (72, 73). While interactions between NI and phenolic compounds against *L. monocytogenes* have rarely been studied in smoked seafood products, synergistic effects between them have been observed for growth of *L. monocytogenes* in carrot juice (74) as well as for the survival of *Pediococcus pentosaceus* NCDO 813 in wine by Knoll et al. (75). Thomas and Isak (76) also demonstrated synergy between NI and the herb rosemary containing high levels of antioxidant phenolic diterpenes against *L. monocytogenes and Bacillus cereus* in chicken soup, meat and cheese pasta sauces. Therefore, phenolic compounds can potentially be a significant factor influencing the antimicrobial effects on cold smoked salmon compared to in BHI broth. Additionally, competition from background microbiota in a food matrix, which would be mainly lactic acid bacteria on cold smoked salmon, may need to be taken into consideration when comparing the effect of antimicrobials in BHI broth and on cold-smoked salmon. Antimicrobials may influence the growth of background microbiota thereby changing the potential interaction between the background microbiota and *L. monocytogenes*. Overall, to fully understand the

mechanism of antimicrobials in a food matrix, further study is needed to investigate the impact of antimicrobials on the native microbiota as well as the pathogen.

CONCLUSION

Data presented here suggest that strain variability, genetic lineage and matrix (BHI versus cold-smoked salmon) can significantly affect the efficacy of NI and organic acids against *L. monocytogenes*. While not quantitatively predictive, data from BHI broth can be used to efficiently identify possible additive, synergistic, or antagonistic interactions between antimicrobials on cold-smoked salmon. Correlations between the characteristics of the growth of *L. monocytogenes* in BHI and on salmon treated with antimicrobials are strong for lineage II strains but weak for lineage I strains, revealing that data from BHI broth cannot accurately predict the variability of antimicrobial effects on cold smoked salmon for all *L. monocytogenes* strains. Growth parameter data collected in the present study, when combined with genetic and cellular level studies, could provide an improved mechanistic understanding of the cellular response and resistance development of *L. monocytogenes* to different antimicrobials.

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CHAPTER 4

TRANSCRIPTOMIC ANALYSIS OF *LISTERIA MONOCYTOGENES* ADAPTATION TO GROWTH ON VACUUM-PACKED COLD SMOKED SALMON

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ABSTRACT

The foodborne pathogen *Listeria monocytogenes* is able to survive and grow in ready-to-eat foods, where it is likely to experience a number of environmental stresses due to refrigerated storage and the physicochemical properties of the food. Little is known about the specific molecular mechanisms underlying survival and growth of *L. monocytogenes* under different complex conditions on/in specific food matrices. RNA-seq was used to understand the transcriptional landscape of *L. monocytogenes* strain H7858 grown on cold-smoked salmon (CSS, water-phase salt 4.65%, pH 6.1) relative to in modified brain heart infusion broth (MBHIB, water-phase salt 4.65%, pH 6.1) at 7^{o}C. Significant differential transcription of 149 genes was observed (FDR<0.05, fold change ≥ 2.5), and 88 and 61 genes were up- and down regulated in H7858 grown on CSS relative to in MBHIB. In spite of these differences in transcriptomes under these two conditions, growth parameters for *L. monocytogenes* were not significantly different between CSS and MBHIB, indicating that the transcriptomic differences reflect how *L. monocytogenes* is able to facilitate growth under these different conditions. Differential expression analysis and Gene Ontology enrichment analysis indicated that genes encoding proteins involved in cobalamin biosynthesis as well as ethanolamine and 1,2-propanediol utilization, have significantly higher transcript levels in H7858 grown on CSS compared to in MBHIB. Our data identify specific transcriptional profiles of *L. monocytogenes* growing on

vacuum-packaged CSS, which may provide targets for development of novel and improved strategies to control *L. monocytogenes* growth on this RTE food.

INTRODUCTION

Listeria monocytogenes is a psychrotolerant foodborne pathogen that causes a potentially severe disease, listeriosis. This pathogen is of particular concern to the ready-to-eat (RTE)-meat and -seafood industries due to its ability to grow at temperatures as low as -0.4°C and salt contents as high as 25% (at 4°C) (1-3). Cold smoked salmon (CSS), a RTE seafood, represents a typical food product that can support the growth of *L. monocytogenes* from low numbers to potentially hazardous levels (4-9). The heat treatment applied during processing of CSS is not sufficient to inactivate microbes present on the raw material, including *L. monocytogenes* (10, 11). In addition, RTE food products, including CSS, can be contaminated with *L. monocytogenes* from environmental sources in processing facilities (10-13). Importantly, typical product characteristics of CSS including pH, water activity, salt, and phenolic components do not seem to be sufficient to control the growth of *L. monocytogenes* if it is present (8, 9).

With the aim of developing control strategies that prevent or reduce growth of this pathogen in RTE seafood products, there is a need for a better understanding of the mechanisms that *L. monocytogenes* uses to survive and grow under the complex conditions on/in specific food matrices. Characterization of bacterial gene expression patterns in different environments can be used to characterize the physiological state of *L. monocytogenes* under different conditions, and to help identify the metabolic pathways that are important for survival and growth of *L. monocytogenes* in food products. This will facilitate identification of new compounds that could specifically interfere with these metabolic pathways and thereby control the growth of *L. monocytogenes* (14). Extensive studies on the transcriptome of *L. monocytogenes* have been conducted to assess how it responds to physical, chemical, or biological stresses that it may

encounter on/in food matrices (15-22). The majority of data from these experiments are based on exposure of *L. monocytogenes* to specific stresses in laboratory medium, providing information about specific stress responses and transcriptional profiles in a controlled environment. This information may not provide the full extent of the bacterial transcriptional landscape in a more complex environment, such as a food matrix.

We characterized the transcriptome of late-log-phase *L. monocytogenes* strain H7858 (a serotype 4b lineage I strain) grown on CSS and the same strain grown to late-log-phase in modified brain heart infusion broth (MBHIB, water-phase (w.p.) salt 4.65%, pH 6.10). While the two conditions chosen here are distinct, they do facilitate characterization of the *L. monocytogenes* transcriptional landscape in a real food as well as comparisons against commonly used reference conditions. Our approach is similar to studies that provided significant insights into the pathogen transcriptional landscape in human or animal hosts, which also, by necessity, must choose reference conditions (e.g., growth in rich media) that differ by a multitude of factors from complex host-associated environments (e.g., presence in intestinal lumen or human blood). For example, previous characterization of the *L. monocytogenes* transcriptome in BHI (designated as the "reference condition" by the authors) and physiologically relevant conditions (e.g. stationary phase, low temperature) as well as in the intestinal lumen of infected mice and inoculated human blood provided crucial knowledge of the *L. monocytogenes* transcriptional landscape under various conditions that this pathogen may experience during transmission (23). In the present study, we demonstrate the use of RNA-seq technology to study global gene expression of *L. monocytogenes* in a RTE seafood product. We overcame the technical difficulties associated with isolating high quality bacterial RNA from the seafood matrix, and took advantage of the probe- and annotation-independence of RNA-seq technology to explore

the genome-wide transcriptional landscape of *L. monocytogenes* grown under the complex conditions on this food matrix.

MATERIALS AND METHODS

Bacterial strain and inoculum preparation. *L. monocytogenes* strain H7858 was used in this study (24). H7858 is a lineage I, serotype 4b strain (representing epidemic clone II), isolated from RTE meat and was linked to a multistate listeriosis outbreak from 1998 to 1999 (24, 25). We selected H7858 for this study as we have studied the phenotypic and transcriptomic responses of this strain to a number of stresses relevant to food products, including organic acids and bactericidal additives, and know that it can grow to high levels on cold smoked salmon (26-28). *L. monocytogenes* H7858 was streaked from frozen BHI stock stored at -80°C in 15% glycerol onto a BHI agar plate, followed by incubation at 37°C for 24 h. A single colony was subsequently inoculated into 5 ml of BHIB (in 16 mm tubes), followed by incubation at 37^{o}C with shaking (230 rpm) for 18 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 18 h, 50 µl BHI culture was inoculated into 5 ml chemically defined medium (DM) (29) and grown to stationary phase in DM at 16°C statically, as described previously (30). This culture was used to inoculate CSS and MBHIB as detailed below. DM was used to approximate a nutrient-limited environment (e.g., food processing plants) that *L. monocytogenes* may encounter before contaminating food.

Growth conditions in BHI and on salmon. BHIB was modified to have 4.65% w.p. NaCl and pH 6.1 to simulate the levels typically present in commercially processed CSS (30). The stationary-phase DM culture was used to inoculate 100 ml of MBHIB in 300-ml Erlenmeyer

culture flasks with metal caps (Bellco Glass Co., Vineland, NJ), with an initial population of approximately 1 x 10^6 CFU/ml, followed by static incubation at 7°C.

Commercially produced wet-cured CSS fillets were stored at -20°C and thawed at 4°C overnight. A mixture of natural hardwood and fruitwood had been used to cold smoke the salmon. The background microbiota (mainly lactic acid bacteria) and physico-chemical characteristics of the untreated salmon slices used in the present study have been described previously by Kang et al. (31). All CSS samples were from the same batch of product. The concentration of lactic acid bacteria on un-inoculated CSS was ~ 4 log(CFU/g) on day 0, ~ 6 log(CFU/g) on day 5, and ~7 log(CFU/g) on day10 during incubation of the vacuum-packaged slices at 7°C. For the un-inoculated CSS, the pH was ~ 6.18, water activity (a_w) was 0.96, moisture content was ~63.58%, fat content was ~8.87% (31). Un-inoculated salmon samples were plated onto Oxford agar and incubated at 30 °C for 48 h to confirm the absence of *L. monocytogenes*.

Salmon slices were weighed (10 ± 0.5 g each) and transferred into sterile petri dishes. Both sides of the salmon slice were inoculated with 500 µl stationary-phase cultures from DM that were diluted in 0.1% sterile peptone water to a target population of approximately 1 x 10^6 CFU/g, and spread with sterile plastic cell spreaders. Inoculated salmon slices were then stored in a biosafety cabinet for 15 min to dry the surface before being transferred into storage bags (oxygen permeability 38.10 cc/m^2 - 40.50 cc/ m^2 at 23°C dry/24 hrs) and packaged using a commercial vacuum sealer (FoodSaver, model V2244). All samples were stored at 7°C. Incubator temperature was recorded every 20 min by an automated thermal recorder during the storage of

both MBHIB cultures and CSS samples. The range of recorded incubation temperature was 7.0 ± 0.5°C.

Determination of exponential-phase sampling points. To monitor *L. monocytogenes* growth, cell density was determined every day, starting from day 0, until log-phase cells were collected for RNA extraction on day 7; another three time points were taken on day 10, 11 and 12 after RNA extraction to determine the maximum cell density. For MBHIB samples, cultures were diluted with 0.1% sterile peptone water and spiral-plated in duplicate onto BHI agar using an Autoplate 4000 (Spiral Biotech, Inc., Norwood, MA). BHI plates were incubated at 37^{o}C for 24 h before colonies were counted with the Q- Count Colony Counter (Spiral Biotech). For salmon samples, 2 vacuum-packed samples were aseptically opened for each time point and stomached for 30 s at high speed setting (Stomacher 400, Seward, West Sussex, UK) with 40 ml of 0.1% sterile peptone water transferred into each bag. CSS homogenates were spiral-plated on Oxford agar (cat. no. 222530, BD; cat. no. SR0140, Oxoid, Ltd., Hampshire, UK) using the Autoplate 4000.

Measurements of *L. monocytogenes* cell density over time in MBHIB and on CSS were fitted with a three-phase linear model described by Buchanan et al. (32) using the NLStools package (v0.0-11) in R v 2.13.0. Four growth parameters including lag phase (λ, [day]), maximum growth rate (μ_{max}, [log(CFU/ml or g/)day]), initial cell density ($N_{0,}$ [log(CFU/ml or g]) and maximum cell density (N_{max}, [log(CFU/ml or g)]) were calculated. Extracted RNA was considered to be qualified for downstream processing and analysis if it had a RNA integrity number (RIN) ≥ 7 (33). Pilot experiments showed that *L. monocytogenes* cell density on CSS needed to be at least 8 log(CFU/g) to obtain total RNA with RIN ≥ 7; at this cell density H7858

was still in log phase. To predict the time point for RNA isolation ($T_{RNA\text{-}extraction}$) (when *L. monocytogenes* cell density reaches 8 log(CFU/ml or g)), we obtained the growth parameters by fitting growth data collected from day 0 to 5 as described earlier and calculated $T_{RNA\text{-}extraction}$ using the equation derived from Buchanan model (32): $T_{RNA\text{-}extraction} = \lambda + (1 \times 10^8 \text{ CFU/g} - N_0) \div \mu_{max}$. The real N_{max} of each growth experiment was confirmed by sampling on day 10, 11 and 12 after RNA extraction to ensure accuracy of the prediction. The time points for RNA extraction fell on day 7 for all four replicates of both CSS and BHI samples.

RNA isolation, integrity and quality assessment. Similar procedures were used to extract RNA from *L. monocytogene*s growing in MBHIB and on CSS. For MBHIB, a total of 7 ml of RNA Protect reagent (Qiagen, Valencia, CA) was added to 7 ml samples in 14 ml Sarstedt tubes (Sarstedt, Nümbrecht, Germany), followed by vortexing vigorously for 10 s to mix well; for CSS, 17 ml of RNA Protect reagent was added to 10 g samples, followed by massaging the sample bag to mix well. The mix was incubated at room temperature for 10 min to ensure that the bacterial RNA was stabilized. For CSS samples, the liquid part of the mix was then filtered out with a 207-ml filter bag (cat. no. B01385WA, Whirl-Pak bag, Nasco, Fort Atkinson, WI) to remove salmon particles. Cells were pelleted by centrifugation (4,637 × *g*, 15 min) at 4°C and suspended in nuclease free water with proteinase K (12.5 mg/ml) and lysozyme (25 mg/ml), followed by incubation at 37°C for 30 min. TRI reagent (Ambion, Austin, TX) was then added to each sample (in screw cap tubes with 3 ml of 0.1-mm acid-washed zirconium beads), followed by mechanical lysis for 5 min in a Mini-Beadbeater-8 (BioSpec Products, Inc., Barlesville, OK) and subsequent RNA extraction according to manufacturer's recommendations. Total RNA was incubated with RQ1 DNase (Promega, Madison, WI) in the presence of RNasin (Promega) to remove remaining DNA. Subsequently, RNA was purified using two phenol-chloroform

extractions and one chloroform extraction, followed by RNA precipitation and suspension of the RNA in RNase free TE (10 mM Tris, 1 mM EDTA; pH 8.0; Ambion). UV spectrophotometry (Nanodrop, Wilmington, DE) was used to quantify and assess purity of the RNA. Efficacy of the DNase treatment was assessed by TaqMan qPCR analysis of DNA levels for the housekeeping gene *rpoB* (34). qPCR was performed using Taq-Man One-Step RT-PCR Master Mix Reagent and the ABI Prism 7000 Sequence Detection System (all from Applied Biosystems, Foster City, CA). All samples showed Ct values > 35 for *rpoB*, indicating negligible levels of DNA contamination. As a final step, RNA integrity was assessed using the 2100 Bioanalyzer (Agilent, Foster City, CA). All experiments were performed in four biological replications.

Preparation of cDNA fragment libraries and RNA-Seq. Preparation of cDNA fragment libraries was performed using the ScriptSeq Complete Kit (Bacteria)-Low Input Kit (Epicentre, Madison, WI). To remove 16S and 23S rRNA from total RNA and enrich for mRNA, 1µg total RNA was treated with Ribo-Zero rRNA Removal Reagents (Bacteria)-Low Input and Magnetic Core Kit-Low Input according to the manufacturer's protocol. Enriched mRNA samples were run on the 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA) to confirm reduction of 16S and 23S rRNA and purified using Agencourt RNAClean XP Kit (Beckman Coulter Inc, Brea, CA) prior to preparation of cDNA fragment libraries. The mRNA-enriched fraction was converted to indexed RNA-seq libraries with the ScriptSeq v2 RNA-Seq Library Preparation Kit. This protocol allows the identification of the specific strand from which each read was generated, resulting in a strand-specific analysis of the RNA-seq results. Indexed and purified libraries (8 libraries including 4 replicates for each CSS and MBHIB) were loaded together onto a flow cell without any other samples; sequencing was carried out on a Hiseq 2000 (single-end, 100-bp per read). RNA-Seq data is available in the NCBI GEO Short Read Archives (GSE64353).

RNA-Seq alignment and coverage. As the H7858 genome has not been completely closed (Genbank accession: AADR00000000), the sequence reads were aligned to a H7858 pseudochromosome. The pseudochromosome was created through alignment of the contigs of the H7858 draft genome to the completely closed genome of the *L. monocytogenes* strain EGD-e (GenBank accession: NC_003210) and subsequent concatenation of these contigs in to a pseudochromosome. Alignment of reads was carried out using the BWA mem algorithm in BWA version 0.7.3a (35). Default parameters were used for the alignment. Coverage at each base position along the chromosome was calculated by enumerating the number of reads that aligned to a given base for each DNA strand separately.

Differential expression analysis. Differential expression of genes under the conditions in MBHIB and on CSS was statistically assessed using the BaySeq method (36) implemented in the **BaySeq 1.16.0 package** available from Bioconductor. This package implements a full Bayesian model of negative binomial distributions to simultaneously assess the likelihood of various models, each representing a possible pattern of expression for a given gene. Library sizes were normalized using the approach described by Bullard et al. (37). To allow for quantitative comparisons among genes and treatments, we used the average normalized RNA-seq coverage (NRC) generated by BaySeq for each gene of the four replicates to identify the genes with highest average NRC of *L. monocytogenes* grown on CSS. Genes were considered differentially expressed if they showed a false discovery rate (FDR) < 0.05 and a fold change (FC) ≥ 2.5 (for genes upregulated on CSS) or FC ≤ 0.4 (for genes downregulated on CSS), where FC = average NRC(CSS) ÷ average NRC(MBHIB). To confirm annotation of differentially expressed genes, the NCBI BLAST standalone program was used to search the H7858 amino acid sequence for each of these genes against the amino acid sequences of the CDS of *L. monocytogenes* strains

EGD-e (GenBank accession: **NC_003210**), 10403S (GenBank accession: **NC_017544**) and F2365 (GenBank accession: **NC_002973**), as well as *Salmonella enterica* Typhimurium LT*2* (GenBank accession: **NC_003197**) *and Escherichia coli* K-12 MG1655 (GenBank accession: **NC_000913**).

GO enrichment analysis. Rather than validating the upregulation of individual genes, we focused on identifying the metabolic pathways that contained multiple differentially expressed genes, by using the results generated by Gene Ontology (GO) enrichment analysis. This method allowed us to statistically confirm the upregulation of specific metabolic pathways that may facilitate the survival and growth of *L. monocytogenes* on CSS. Enrichment of GO terms among genes upregulated on CSS was assessed using the GOseq 1.18.0 package (38) available from Bioconductor. GO term classification for each gene in H7858 was obtained using the blast2go program (https://www.blast2go.com).

RESULTS

***L. monocytogenes* growth parameters are similar on CSS and in MBHIB.** *L. monocytogenes* growth parameters, including λ, μ_{max}, N_0, and N_{max}, did not differ significantly between CSS and MBHIB ($p>0.05$; t-test) (Fig. 4.1). The average λ was 1.78 ± 1.05 and 2.12 ± 0.35 days for CSS and MBHIB, respectively. The average μ_{max} was 0.35 ± 0.06 log(CFU/g)/day and 0.40 ± 0.02 log(CFU/ml)/day for CSS and MBHIB, respectively. The average N_0 and average N_{max} were 6.48 ± 0.16 and 8.68 ± 0.14 log(CFU/g) for CSS, respectively, and 6.35 ± 0.06 and 8.82 ± 0.07 log(CFU/ml) for MBHIB, respectively.

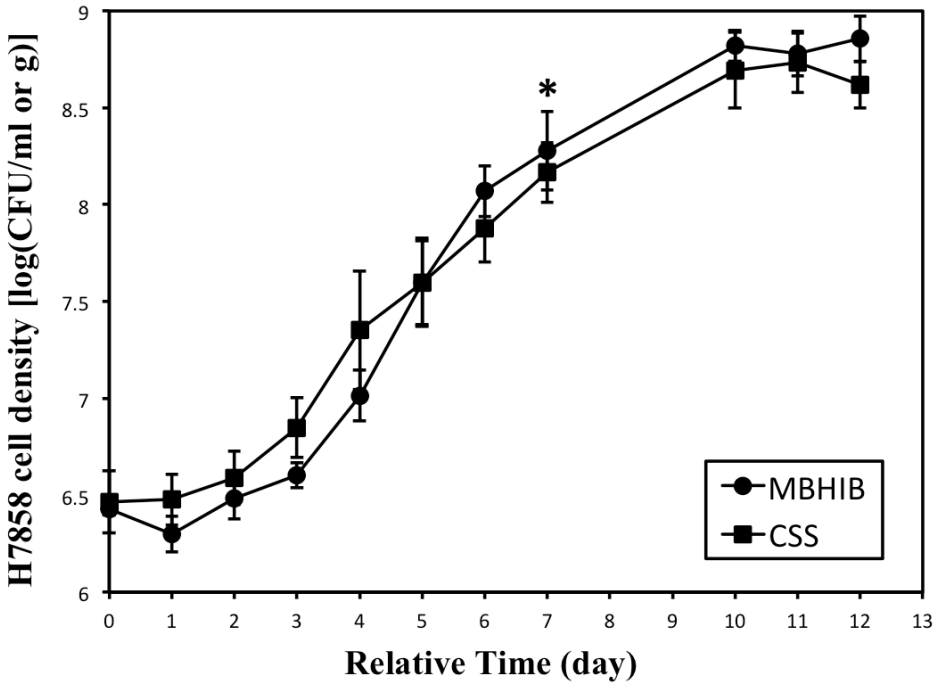

Figure 4.1. Growth of H7858 on cold smoked salmon and in modified BHI broth

Growth of H7858 on cold smoked salmon (CSS; ■) and modified BHI broth (w.p. salt 4.65%, pH 6.10) (MBHIB; •) at 7°C. RNA was extracted at the average H7858 cell density of 8.17 ± 0.16 log(CFU/g) on CSS and 8.28 ± 0.21 log(CFU/ml) in MBHIB (*).

Late log-phase H7858 has 88 up- and 61 downregulated genes on CSS compared to in MBHIB. RNA-seq was performed on H7858 RNA samples representing four independent biological replicates of H7858 grown on CSS or in MBHIB. Samples for RNA isolation were collected when H7858 was grown to late log phase under the conditions of these two matrices; the average cell density of collected *L. monocytogenes* samples were 8.17 ± 0.16 log(CFU/ml) for CSS and 8.28 ± 0.21 log(CFU/g) for MBHIB (Fig. 4.1). Since the growth parameters of H7858 were not significantly different on CSS and in MBHIB, the *L. monocytogenes* cells from both conditions at the time point for RNA isolation were expected to be at the same growth phase, indicating that observed differences in transcript levels were not likely to reflect different growth phases of H7858. Transcriptome sequencing generated 1.6 to 10.9 million reads per sample (Table 4.1). For RNA samples from H7858 grown in MBHIB and on CSS, of the reads that mapped to the reference pseudochromosome, ~ 80% and ~82% of them mapped to protein coding sequences, respectively. The remaining reads mapped to non-coding RNA, including ribosomal RNA and tRNAs. Among the unmapped reads from CSS, 83% on average were found to represent sequences that mapped to Atlantic Salmon genomic DNA (GenBank accession: AGKD00000000.3), suggesting contamination with residual salmon RNA, and 6% on average mapped to the genome of *Carnobacterium maltaromaticum* LMA28 (GenBank accession: NC_019425.2), which is representative of a genus of gram-positive bacteria that is found in food products and grows anaerobically (39), and likely represents part of the resident microbiota of CSS.

Table 4.1. Summary of RNA-seq coverage data

Sample ID	No. of mapped reads	No. of reads mapped to CDS[c]	Percentage of reads mapped to CDS
MBHIB[a]-1	1,555,154	1,293,750	83%
MBHIB-2	2,020,923	1,684,965	83%
MBHIB-3	6,182,126	4,828,777	78%
MBHIB-4	6,302,268	4,821,223	76%
Average MBHIB	4,015,118	3,157,179	80%
CSS[b]-1	1,611,849	1,314,001	82%
CSS-2	2,303,793	1,946,859	85%
CSS-3	3,557,097	2,830,220	80%
CSS-4	3,204,674	2,684,028	84%
Average CSS	2,669,353	2,193,777	82%

[a]MBHIB, modified brain heart infusion broth, water phase salt 4.65%, pH 6.10;
[b]CSS, cold smoked salmon;
[c]CDS, coding DNA sequence for protein

As RNA-seq allows for absolute quantification, our data allowed us to identify the genes that showed the highest transcript levels in H7858 on CSS (Table 4.2). The three genes with the highest average NRC were *fusA*, *eno*, and *tuf*, which encodes translation elongation factor G, an enolase, and translation elongation factor Tu, respectively. Other genes with well-defined functions and high average NRC include *gadT2D2* (40), which encode proteins involved in glutamate dependent acid resistance, *gap*, which encodes a NAD-dependent glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis, and *cspLA*, which encode cold-shock proteins involved in adaptation to atypical conditions (Table 4.2).

Table 4.2. Genes with highest average normalized RNA-seq coverage (NRC)

Gene name in			H7858 Gene Product	Average NRC rank for		Average NRC[b] for	
H7858	EGD-e [a]	10403S [a]		CSS	MBHIB	CSS	MBHIB
LMOh7858_2915	lmo2654 (*fusA*)	LMRG_02199	Translation elongation factor G	1	1	3,643,359	3,404,382
LMOh7858_2604	lmo2455 (*eno*)	LMRG_01793	Enolase (EC 4.2.1.11)	2	3	3,348,509	2,505,110
LMOh7858_2914	lmo2653 (*tuf*)	LMRG_02198	Translation elongation factor Tu	3	4	3,296,021	2,329,659
LMOh7858_2506	lmo2363 (*gadD2*)	LMRG_01479	Glutamate decarboxylase (EC 4.1.1.15)	4	65	3,186,282	627,851
LMOh7858_2608	lmo2459 (*gap*)	LMRG_01789	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	5	15	2,650,840	1,618,077
LMOh7858_2505	lmo2362	LMRG_01480	Probable glutamate/gamma-aminobutyrate antiporter	6	86	2,589,714	490,907
LMOh7858_1751	lmo1634	LMRG_01332	Alcohol dehydrogenase (EC 1.1.1.1); Acetaldehyde dehydrogenase (EC 1.2.1.10)	7	8	2,504,749	1,849,977
LMOh7858_2605	lmo2456 (*gpmI*)	LMRG_01792	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1)	8	12	2,229,167	1,648,039
LMOh7858_2609	lmo2460	LMRG_01788	Central glycolytic genes regulator	9	32	2,197,665	1,008,437
LMOh7858_2607	lmo2458 (*pgk*)	LMRG_01790	Phosphoglycerate kinase (EC 2.7.2.3)	10	15	2,158,721	1,592,390
LMOh7858_2353	lmo2219 (*prsA2*)	LMRG_01613	Foldase protein PrsA precursor (EC 5.2.1.8) @ Foldase clustered with pyrimidine conversion	11	2	1,919,848	2,662,595
LMOh7858_2899	lmo2638	LMRG_02183	pyridine nucleotide-disulfide oxidoreductase family protein	12	665	1,913,850	1,275,847
LMOh7858_2708	lmo2556 (*fbaA*)	LMRG_01691	Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	13	1,378	1,892,452	951,955

Table 4.2 (Continued)

LMOh7858_2043	lmo1917 (*pflA*)	LMRG_01064	Pyruvate formate-lyase (EC 2.3.1.54)	14	19	1,886,299	1,435,588
LMOh7858_1070	lmo1003 (*ptsI*)	LMRG_02103	Phosphoenolpyruvate-protein phosphotransferase of PTS system (EC 2.7.3.9	15	6	1,810,680	1,933,477
LMOh7858_1451	lmo1364 (*cspLA*)	LMRG_00814	cold-shock protein	16	67	1,723,202	615,514
LMOh7858_1331	lmo1250	LMRG_00696	Transporter	17	250	1,711,134	210,841
LMOh7858_0642	lmo0582 (*iap*)	LMRG_00264	P60 extracellular protein, invasion associated protein Iap	18	5	1,675,726	2,318,702
NGN[c]	lmo1388 (tcsA)	LMRG_00840	Unspecified monosaccharide ABC transport system, substrate-binding compo	19	9	1,607,769	1,818,968
LMOh7858_2330	lmo2196 (oppA)	LMRG_01636	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein O	20	10	1,603,950	1,694,246

[a]Strain EGD-e (GenBank accession no.: NC_003210) and 10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;
[b]NRC, normalized RNA-seq coverage; genes with average NRC >1,500,000 are presented;
[c]NGN, no gene name given; in the published version of the H7858 genome (27), this gene was not annotated and, therefore, did not receive a locus name; the gene was identified in our annotation.

Initial analysis of the RNA-seq data identified 88 and 61 genes that showed significantly higher and lower transcript levels, respectively, for H7858 grown on CSS compared to in MBHIB (see Tables S4.1 and S4.2 for lists of up- and downregulated genes). The 88 upregulated genes included genes encoding proteins annotated as being involved in cobalamin biosynthesis (26 genes), ethanolamine utilization (8 genes), 1,2-propanediol utilization (7 genes), carbohydrate transport and utilization (14 genes), the non-oxidative branch of the pentose pathway (5 genes), agmatine deiminase (4 genes), as well as genes regulated by PrfA (5 genes) (Tables S4.3-S4.7). The 61 downregulated genes included genes encoding proteins annotated as being involved in pyrimidine nucleotide biosynthesis (6 genes) and L-cystine ABC transporter (3 genes) (Table S4.2). GO enrichment analysis identified 37 GO terms that were overrepresented among genes upregulated in *L. monocytogenes* grown on CSS, as compared to MBHIB (Table 4.3). No GO terms were found to be enriched among the downregulated genes.

Table 4.3. GO terms enriched among genes upregulated in H7858 grown on CSS compared to in MBHIB at 7°C

GOID	GO Term	Pathways associated with GO term		
		cobalamin biosynthesis /transport	ethanolamine/1,2-propanediol utilization	carbohydrate utilization/ transport
GO:0005363	maltose transmembrane transporter activity			Y[a]
GO:0006580	ethanolamine metabolic process		Y	
GO:0006766	vitamin metabolic process	Y		
GO:0006767	water-soluble vitamin metabolic process	Y		
GO:0006778	porphyrin-containing compound metabolic process	Y		
GO:0006779	porphyrin-containing compound biosynthetic process	Y		
GO:0006824	cobalt ion transport	Y		
GO:0008757	S-adenosylmethionine-dependent methyltransferase activity	Y		
GO:0009110	vitamin biosynthetic process	Y		
GO:0009235	cobalamin metabolic process	Y		
GO:0009236	cobalamin biosynthetic process	Y		
GO:0015151	alpha-glucoside transmembrane transporter activity			Y
GO:0015157	oligosaccharide transmembrane transporter activity			
GO:0015235	cobalamin transporter activity	Y		
GO:0015420	cobalamin-transporting ATPase activity	Y		
GO:0015422	oligosaccharide-transporting ATPase activity			Y

Table 4.3 (Continued)

GO:0015423	maltose-transporting ATPase activity			Y
GO:0015768	maltose transport			Y
GO:0015889	cobalamin transport	Y		
GO:0015994	chlorophyll metabolic process	Y		
GO:0016628	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	Y		
GO:0022804	active transmembrane transporter activity	Y	Y	
GO:0033013	tetrapyrrole metabolic process	Y		
GO:0033014	tetrapyrrole biosynthetic process	Y		
GO:0034311	diol metabolic process		Y	
GO:0034313	diol catabolic process		Y	
GO:0042364	water-soluble vitamin biosynthetic process	Y		
GO:0042439	ethanolamine-containing compound metabolic process		Y	
GO:0042440	pigment metabolic process	Y	Y	
GO:0044237	cellular metabolic process	Y	Y	Y
GO:0046483	heterocycle metabolic process	Y	Y	
GO:0051143	propanediol metabolic process		Y	
GO:0051144	propanediol catabolic process		Y	
GO:0051180	vitamin transport	Y		
GO:0051183	vitamin transporter activity	Y		
GO:0051186	cofactor metabolic process	Y		
GO:0051188	cofactor biosynthetic process	Y		

[a]Y, yes.

Genes involved in cobalamin synthesis, ethanolamine utilization, and 1,2-propanediol utilization were upregulated in H7858 growing on CSS. In addition to identification of 26 upregulated genes with cobalamin metabolism related annotations (Table S4.3), we also found the GO terms "cobalamin biosynthetic process" and "cobalamin transport" to be overrepresented among upregulated genes of H7858 on CSS (Table 4.3). We mapped these genes to the overall cobalamin biosynthesis pathways (Fig. 4.2) constructed based on the metabolic pathway data of EGD-e and 10403S available in the BioCyc database (biocyc.org) (41). Among the 21 genes mapped to the cobalamin biosynthesis pathways (Fig. 4.2), 18 were found to be upregulated (FC 2.76-12.88).

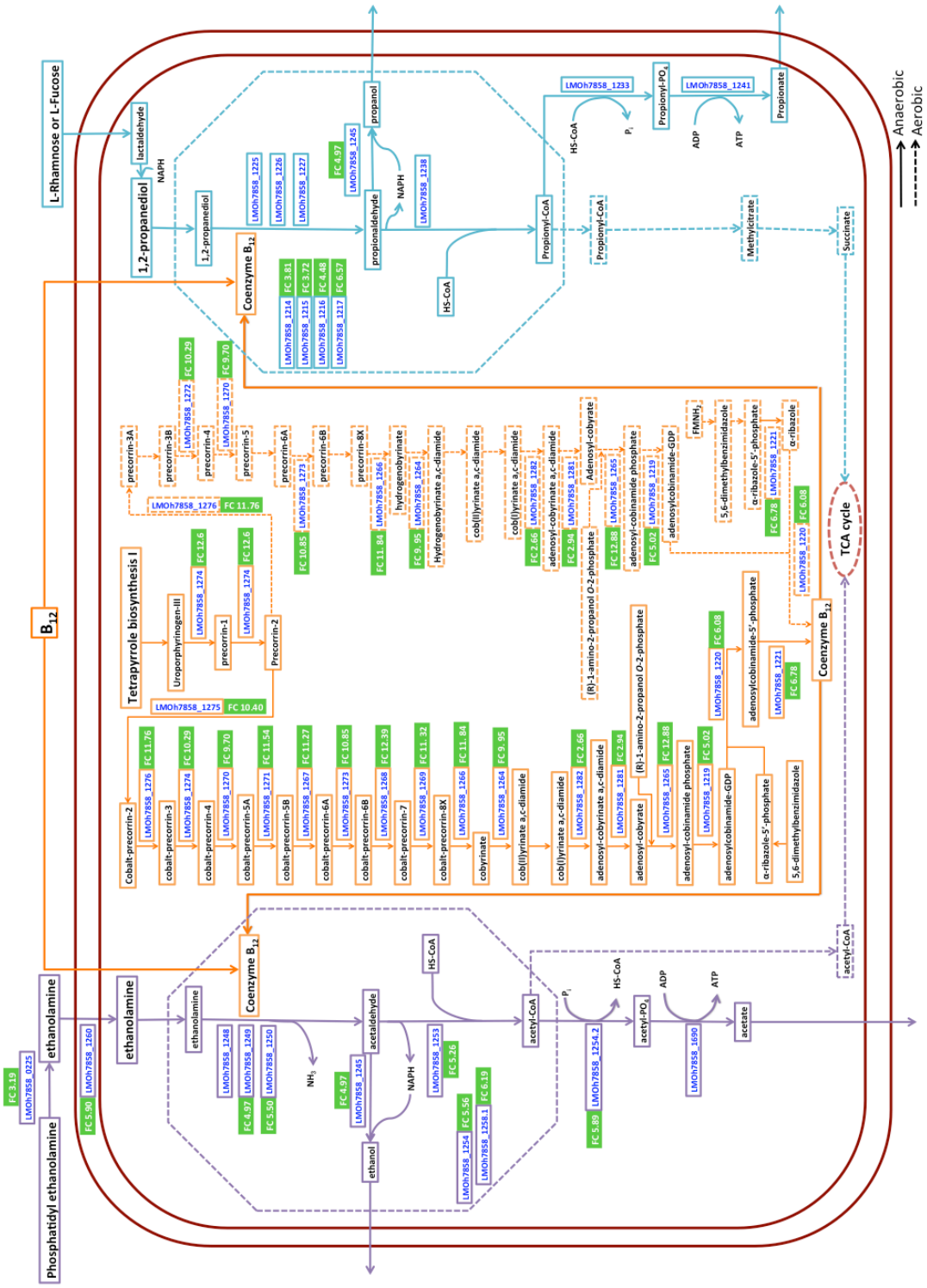

Figure 4.2. Cobalamin biosynthesis, ethanolamine and 1,2-propanediol utilization pathways in H7858

Pathways were constructed based on information provided in the Biocyc database and previous studies (42-44). H7858 protein designations for enzymes involved in these pathways are shown in blue text. Enzymes encoded by genes upregulated in H7858 grown on cold smoked salmon (CSS) compared to in modified BHI broth (MBHIB) are designated by display of the fold change (FC) in green boxes. Microcompartments are depicted by dotted lines. Purple, orange, and blue boxes mark molecules involved in ethanolamine, cobalamin, and 1,2-propanediol metabolism, respectively. Solid and dotted orange boxes mark reactions under anaerobic and aerobic conditions, respectively.

We also identified eight upregulated genes with ethanolamine utilization related annotations (Table S4) and the GO term “ethanolamine metabolic process" as overrepresented among upregulated genes in H7858 grown on CSS compared to in MBHIB (Table 4.3). Mapping of these genes to the ethanolamine utilization pathway, constructed based on previous studies for *S. enterica* (42-44) and information about the *eut* operon provided by Staib and Fuchs (42), showed that genes encoding eight of the eleven key enzymes mapped to the ethanolamine degradation pathway were upregulated (FC 4.97-6.19) (Fig. 4.2). Furthermore, we identified seven upregulated genes with 1,2-propanediol utilization related annotations (Table S4.4) and the GO term “propanediol catabolic process” as overrepresented among upregulated genes (Table 4.3). We mapped these genes to the 1,2-propanediol utilization pathway which was constructed based on data from *S. enterica* (42-44) and information about the *pdu* operon provided by Staib and Fuchs (42). This analysis showed that six of the thirteen genes encoding key enzymes mapped to the 1,2-propanediol degradation pathway (Fig. 4.2) were upregulated on CSS (FC 2.88-6.57).

Genes involved in carbohydrate transport and utilization were upregulated in H7858 growing on CSS. We identified fourteen upregulated genes with carbohydrate and alcohol transport and utilization related annotations (Table S4.5) and the GO terms “alpha-glucoside transmembrane transporter activity” and “oligosaccharide-transporting ATPase activity” were overrepresented among upregulated genes (Table 4.3). As the fourteen upregulated genes represented functions related to transport and metabolism of galactitol, mannose and maltose, we diagrammed the galactitol and mannose specific phosphotransferase system (PTS), maltose specific ATP-binding cassette (ABC) transporter system, as well as a few catabolism reactions for each of these three molecules (Fig. 4.3) to further assess expression of these pathways. For the mannose-specific PTS of *L. monocytogenes*, genes encoding all four components (PTS-

IIA^{Man}, PTS-IIB^{Man}, PTS-IIC^{Man} and PTS-IID^{Man}) were upregulated (Fig. 4.3). For the galactitol-specific PTS, which includes three components, genes encoding two components (PTS-IIB^{Gat} and PTS-IIC^{Gat}) were upregulated. For the maltose-specific ABC transporter system of *L. monocytogenes*, genes encoding all three domains of this ABC transporter (MalE, MalF and MalG) were upregulated.

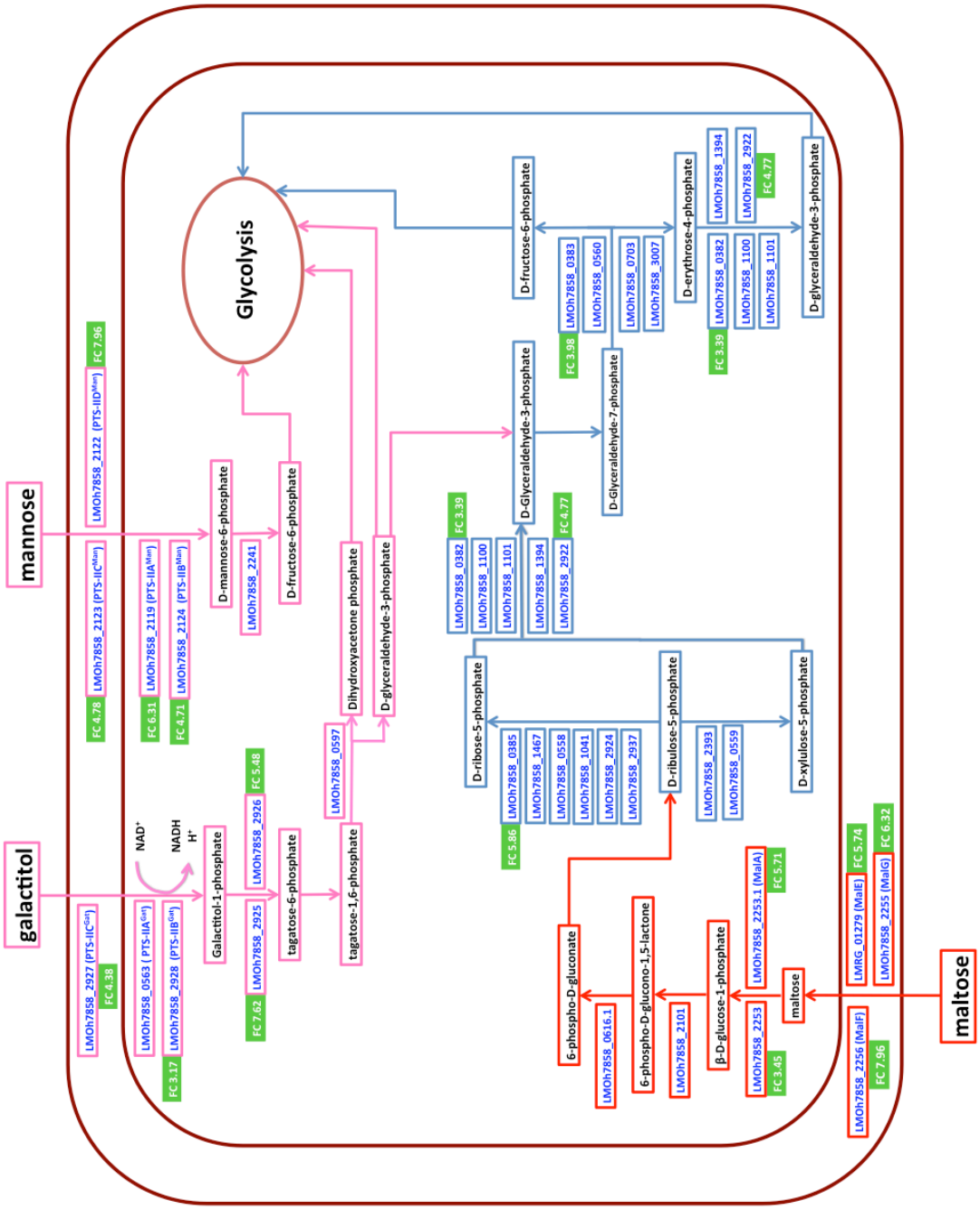

Figure 4.3. Schematic of galactitol and mannose specific phosphotransferase system (PTS), maltose specific ATP-binding cassette (ABC) transporter system, catabolism reactions for each of the three molecules, and the non-oxidative branch of pentose phosphate pathway in H7858

Pathways were constructed based on information provided in the Biocyc database. H7858 protein designations for enzymes involved in these reactions and pathways are shown in blue text. Enzymes encoded by genes upregulated in H7858 grown on cold smoked salmon (CSS) compared to in modified BHI broth (MBHIB) are designated by display of the fold change (FC) in green boxes. Pink, red and blue boxes mark molecules involved in galactitol and mannose specific PTS and catabolism reactions, maltose specific ABC transporter system and catabolism reactions; as well as the non-oxidative branch of pentose phosphate pathway, respectively.

Additionally, we found that four of the sixteen genes involved in the non-oxidative branch of the *L. monocytogenes* pentose phosphate pathway were significantly upregulated in H7858 growing on CSS (Table S4.5); these genes encode enzymes involved in four out of five key reactions of this pathway branch based on the 10403S database on BioCyc (Fig. 4.3). Moreover, 6-phospho-D-gluconate, generated by maltose utilization reactions, and D-glyceraldehyde-3-phosphate, generated from galactitol utilization reactions, are also found to be the participants of the non-oxidative branch of the pentose phosphate pathway in *L. monocytogenes* (Fig. 4.3), which reflect a potential connection between these two carbohydrate utilization pathways and the non-oxidative branch of the pentose phosphate pathway.

Genes involved in agmatine deiminase system were upregulated in H7858 growing on CSS. We also identified four upregulated genes with agmatine deiminase related annotations (Table S4.6); the fold changes of these genes ranged from 13.67 to 31.36 and were the highest among all upregulated genes. To further explore the functions of these genes, we reconstructed the reactions involved in the *L. monocytogenes* agmatine deiminase system, using previous studies on *L. monocytogenes* and other gram-positive bacteria (45-48). All four genes encoding the key enzymes involved in breakdown of agmatine to CO_2 and NH_3 showed higher transcript levels in H7858 grown on CSS (Fig. 4.4), indicating upregulation of the overall agmatine deiminase pathway.

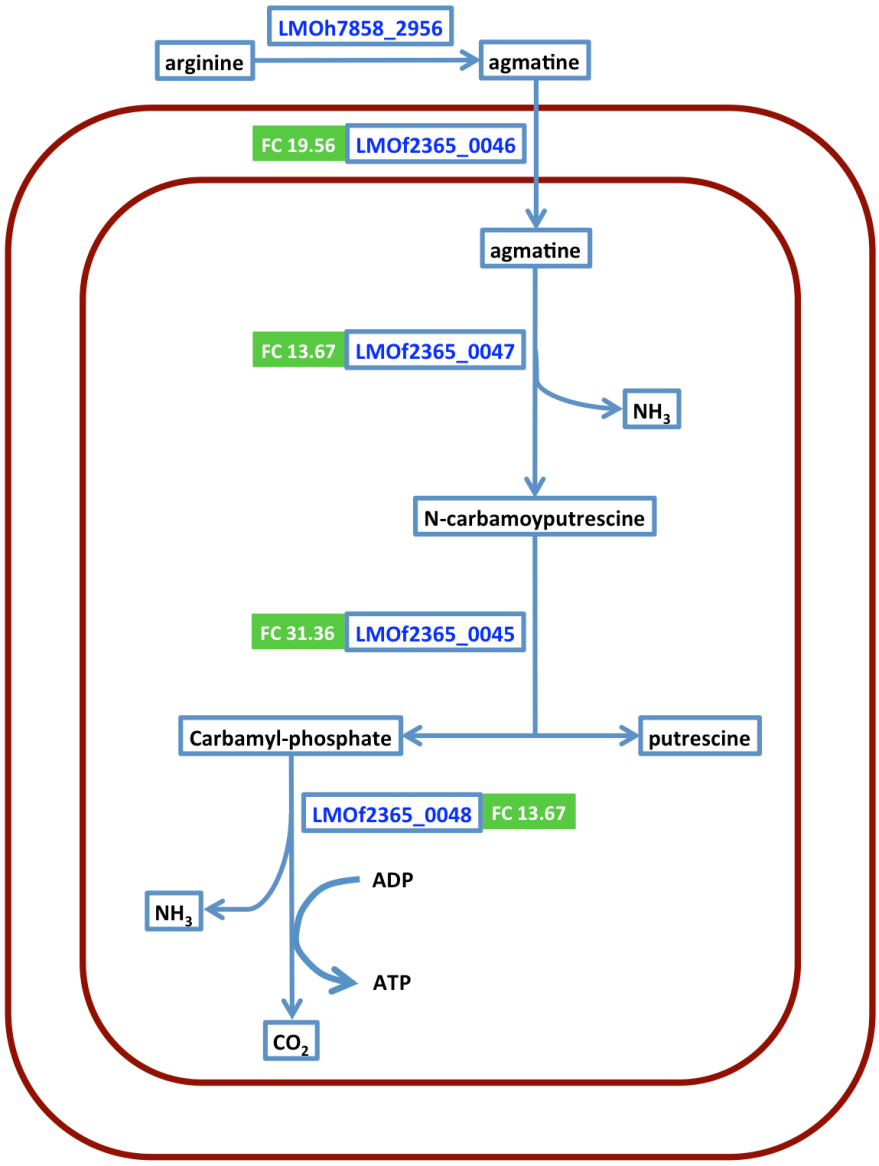

Figure 4.4. Agmatine deiminase system in H7858

Agmatine deiminase system in H7858 was constructed by connecting reactions associated with agmatine deiminase based on previous studies (45-48). H7858 protein designations for enzymes involved in these reactions are shown in blue text. Enzymes encoded by genes upregulated in H7858 grown on cold smoked salmon (CSS) compared to in modified BHI broth (MBHIB) are designated by display of the fold change (FC) in green boxes. Blue boxes mark molecules involved in agmatine deiminase system.

***L. monocytogenes* grown on CSS show higher transcript levels of PrfA-dependent genes.** Five genes known to be regulated by the master regulator of virulence genes, PrfA, were found to be upregulated in H7858 grown on CSS. These PrfA-dependent genes include *inlB* (FC 2.71), *plcA* (FC 2.83), *hly* (FC 2.54), *actA* (FC 2.86), and *plcB* (FC 3.19) (Table S4.7). Statistical analysis showed that PrfA-dependent genes as a group were significantly enriched among upregulated genes ($P < 0.0001$; Fishers exact test).

DISCUSSION

In this study we used RNA-seq to explore the transcriptional landscape of *L. monocytogenes* H7858 growing on CSS and in BHIB modified to reflect the pH and water phase salt concentration of CSS. Our data indicate that H7858 grown on vacuum-packaged CSS (i) upregulates cobalamin biosynthesis pathways as well as ethanolamine and 1,2-propanediol utilization pathways, (ii) differentially regulates carbohydrate transport functions, and (iii) upregulates arginine deiminase genes, likely facilitating adaptation to anaerobic conditions, utilization of nutrients available on CSS and growth in the presence of resident microbiota.

Limitations of using MBHIB as a reference condition to CSS for studying the gene expression profile of *L. monocytogenes* during growth. A number of previous studies have analyzed the transcriptomes of foodborne pathogens under stress conditions commonly present on/in food matrices (including hyperosmotic stress, cold stress, hydrostatic pressure stress, antimicrobial stress, acid stress and alkali stress), using laboratory media modified to simulate these conditions as a model (15-22, 49-63). These laboratory media include BHI broth (18, 20, 21, 59), BHI agar (19), Luria-Bertani broth (60, 61), tryptic soy broth (62), M9-glucose (63), and *Listeria* minimal medium (22), as references conditions. Key differences between the reference

condition we used and CSS were that the CSS was vacuum packaged while the reference medium was not anaerobic (though MBHIB was incubated without shaking), *L. monocytogenes* was grown on the surface of CSS, while growth was planktonic in the liquid MBHIB, and CSS contains a number of unique components that cannot be easily added to BHIB at comparable concentrations (e.g., different phenolics, lipids, trace elements, etc.). In addition, a previous study in our lab on the same batch of CSS demonstrated that the resident lactic acid bacteria were able to grow to ~ 7 log(CFU/g) by day 10 [with an initial density of ~ 4 log(CFU/g) on day 0] under the same growth conditions used in the current study (31). Lactic acid bacteria are known to be one of the dominant microbes on CSS and may constitute a natural form of antimicrobial control via competition for particular nutrients or production of organic acids, hydrogen peroxide, and bacteriocins (64-66). Furthermore, *Lactobacillus* has been demonstrated to be able to reshape *L. monocytogenes* protein-coding genes and sRNA expression profiles (67). Even though we modified the laboratory medium to present the same major conditions found in CSS, and *L. monocytogenes* had similar growth patterns under the conditions of both the food matrix and laboratory medium, the aforementioned differences between these two conditions add limitations to the comparison of the transcriptional profiles.

The inoculation level of *L. monocytogenes* in MBHIB and on CSS was high in the present study (~ 1 x 10^6 CFU/g). *L. monocytogenes* contamination of cold smoked fish is typically at low levels (8, 68), and there is the potential for differences in responses of *L. monocytogenes* when inoculated at different levels. However, *L. monocytogenes* can grow in food products at refrigeration temperatures to high levels, and the infection dose in an immune-competent individual can be high [up to ~9 log(CFU/g)] (69). While our study does not encompass all possible variations in inoculum level, media preparation, and competitive microbes found on

some naturally contaminated products, data from previous studies indicate that what we found was not unusual. Consistent with our results, studies with lower inoculum levels also found similar growth rates (e.g. 0.37 log units/day) and similar final concentrations (e.g. ~8 log CFU/ml) for *L. monocytogenes* on vacuum packed CSS at refrigerated temperatures (e.g. 4^{o}C) (7, 70). Our results do provide insights into the relative differences in transcriptomes of *L. monocytogenes* at the same growth phase under different complex conditions, and reflect the gene expression profile of *L. monocytogenes* in food at high levels that could cause human disease.

***L. monocytogenes* grown on vacuum-packaged CSS upregulates cobalamin biosynthesis pathways as well as ethanolamine and 1,2-propanediol degradation pathways, likely facilitating adaptation to available nutrient sources.** Our data showed that *L. monocytogenes* significantly upregulated both cobalamin biosynthesis and transport systems, presumably to increase the availability of cobalamin in the bacterial cells. Cobalamin (coenzyme B_{12} (71)) is found in high levels in CSS. Smoked salmon has up to 18.10 μg B_{12} per 100 g according to the USDA National Nutrient Database for Standard Reference (Basic Report: 35190, Salmon, red (sockeye), smoked). We found that transcript levels of genes involved in both aerobic and anaerobic pathways (72-74) were significantly higher in *L. monocytogenes* grown on CSS. Roth et al. (43) proposed that the primary function of cobalamin in many bacteria is to support fermentation of small molecules such as ethanolamine and 1,2-propanediol by catalyzing molecular rearrangements. We propose that one of the reasons *L. monocytogenes* uptakes or synthesizes cobalamin more actively on CSS is to facilitate growth under this condition by using cobalamin to support the utilization of ethanolamine and 1,2-propanediol.

To date, three foodborne bacterial pathogens have been shown to be able to use both ethanolamine and 1,2-propanediol as a carbon source (75), and to use ethanolamine as a nitrogen source: *L. monocytogenes*, *S. enterica*, and *Clostridium perfringens* (76). Ethanolamine is a major constituent of lipids in eukaryotic cells (77) including salmon (78), and thus may become available for *L. monocytogenes* through the breakdown of salmon cells. Broad-range phospholipases such as PlcB (*plcB* was upregulated in CSS) of *L. monocytogenes* might serve to reduce phosphatidylethanolamine to ethanolamine (42). 1,2-propanediol is produced during bacterial anaerobic catabolism of the common methylpentoses, rhamnose and/or fucose (42, 79). 1,2-propanediol may be available for *L. monocytogenes* through the breakdown of salmon mucosal glycoconjugates which contain fucose and rhamnose (80, 81). Moreover, in *L. monocytogenes,* one cobalamin-binding riboswitch is located upstream of the first gene in the *eut* locus; this riboswitch regulates expression of *eut* in response to cobalamin availability (82). A second cobalamin-binding riboswitch is located upstream of the *pdu* locus; this riboswitch maximizes the expression of 1,2-propanediol utilization genes when both 1,2-propanediol and cobalamin are present (83). These findings further demonstrate the close relationship between ethanolamine and 1,2-propanediol utilization pathways and cobalamin biosynthesis/transport pathways.

Recent research has identified potential roles for ethanolamine and 1,2-propanediol utilization in *L. monocytogenes* and *S.* Typhimurium during growth in foods and/or in the host environment (84-87). Srikumar and Fuchs (88) found that nonpolar deletions of *pocR* (regulating *pdu* and *cob-cbi*) and *eutR* in *S. enterica* Typhimurium led to significantly reduced proliferation in milk and egg yolk. Likewise, Goudeau et al. (84) reported that *S. enterica* mutants with deletions in *pduD* or *cobS* show decreased fitness in cilantro soft rot. Archambaud et al. (67)

reported that *L. monocytogenes* shows higher transcript levels of genes encoding functions involved in ethanolamine and 1,2-propanediol utilization as well as cobalamin biosynthesis when present in the intestine of gnotobiotic mice that contained *Lactobacillus spp.*, as compared to mice without *Lactobacillus spp.*. This suggests that 1,2-propanediol and ethanolamine utilization may provide *L. monocytogenes* a mechanism to effectively co-exist with the resident microbiota, which are usually not able to utilize these organic compounds. Overall, our study along with other studies suggest that foodborne pathogens including *L. monocytogenes* may utilize ethanolamine and/or 1,2-propanediol to proliferate in food and host environments where these molecules are available. Further studies will be needed to confirm this and to identify food matrices or growth conditions where ethanolamine and/or 1,2-propanediol utilization by *L. monocytogenes* may occur.

Listerial physiological adaptation to the end products of ethanolamine and 1,2-propanediol utilization on CSS may provide targets for novel interventions. Under anaerobic conditions, two of the major products of the ethanolamine and 1,2-propanediol utilization pathways are acetate (42-44, 89) and propionate (42-44, 90), respectively. We propose that *L. monocytogenes* utilizes the agmatine deiminase system to attenuate the acidification caused by these two acids. This is supported by the observation that *L. monocytogenes* grown in vacuum-packaged CSS upregulated genes encoding functions involved in the agmatine deiminase system. This system has been demonstrated to catalyze arginine and/or agmatine deamination, which generates two NH_3 molecules, facilitating pH buffering (45-48, 91) and thus possibly buffering acid end products (acetate and propionate) created by the ethanolamine and 1,2-propanediol degradation. However, the upregulation of genes related to this system have not been found in studies on *L. monocytogenes* growing on/in food matrices such as turkey deli meat (19), skim

milk (21), or cut cabbage (22), which may indicate that this transcriptional response is specific to the growth conditions tested here. Production of acetate and propionate as byproducts of ethanolamine and 1,2-propanediol degradation, suggests that growth inhibitors that include these two organic acids may be able to more effectively inhibit *L. monocytogenes* growth than currently used growth inhibitors. This is supported by a recent transcriptomic study in BHIB grown *L. monocytogenes* (17), which showed that exposure of *L. monocytogenes* to acetate and lactate lead to decreased expression of lactate and acetate creating energy pathways, shifting ATP production to a less efficient pathway with acetoin, a non-charged molecule, as an end product.

Higher transcript levels of genes encoding specific carbohydrate PTS components and ABC transporter domains indicate that *L. monocytogenes* may uptake and utilize a broad range of carbohydrates on CSS. As *L. monocytogenes* upregulated genes encoding proteins involved in utilization and transport of galactitol, mannose and maltose, we propose that *L. monocytogenes* growing on CSS broadens the range of carbohydrates that are utilized to compensate for the limited availability of glucose (relative to BHIB, which contains 2g added glucose/L). Galactitol is the reduction product of galactose, which together with mannose, may be available as components of mucin glycoconjugates at the mucosal tissue of fish (80, 81, 92, 93). While maltose is found in many processed products that have been sweetened (94), it is unclear whether there is a specific maltose source in CSS. Consistent with our findings, genes involved in carbohydrate metabolism were identified to be more expressed on cut-cabbage (22) and on ready-to-eat turkey deli meat (19), as compared to the growth of *L. monocytogenes* in laboratory media. However, no genes involved in carbohydrate metabolism were found to be upregulated in skim milk in Liu and Ream's study (21), which may be attributed to the

abundance of lactose as a carbon source in milk. In the host environment, Toledo-Arana (23) and Chatterjee et al. (95) found increased transcript levels of specific PTSs in *L. monocytogenes*, which enabled it to utilize sugars, such as mannose, fructose, and/or their alcohols including galactitol, during growth in the intestine of axenic mice (23) or upon entering epithelial cells (95). In sum, exploiting a wider range of carbohydrates appears to be a successful strategy of enteric pathogens to overcome nutrient limitations or adapt to specific nutrient compositions when proliferating under the complex conditions of food matrices such as CSS.

Our data suggest that *L. monocytogenes* growing on CSS may use the non-oxidative branch of the pentose phosphate pathway for gluconeogenesis, which is plausible since the pentose phosphate pathway is more efficient in anabolism compared to glycolysis. Similar to our finding, Bae et al. (19) also found the gene LMOf2365_1395 of *L. monocytogenes* F2365 involved in the pentose phosphate pathway was induced on ready-to-eat turkey deli meat (19). Zhou et al. (96) found that several key enzymes involved in the pentose phosphate pathway were more prevalent in biofilms compared to planktonic-grown cells of *L. monocytogenes*, and Hefford et al. (97) reported that proteins involved in sugar metabolism were highly expressed in biofilms. These findings may indicate that upregulation of aforementioned carbohydrate utilization genes, especially the pentose phosphate pathway related genes, is due to growth of *L. monocytogenes* on the surface of foods, such as deli meat and CSS.

Differential regulation of PrfA-dependent genes in vacuum-packaged CSS supports that growth conditions can have multifaceted effects on gene expression and cell physiology. Interestingly, PrfA-dependent genes showed higher transcript levels in vacuum-packaged CSS, which may be triggered by (i) the low level of glucose on CSS, (ii) the oxygen restriction

condition presented in vacuum-packaged CSS, and/or (iii) specific signals associated with salmon tissue or specific compounds in the BHIB that could suppress the expression/activity of PrfA. As the presence of glucose and fermentable carbohydrates can affect expression of the PrfA regulon (98-100), the apparent utilization of different carbohydrates by *L. monocytogenes* grown on CSS may affect the transcript level of PrfA-dependent genes in *L. monocytogenes* grown on CSS. The importance of the anaerobic environment is supported by data from Larsen et al. (101), who reported higher invasion of Caco-2 cells for *L. monocytogenes* grown for two and four weeks on modified atmosphere-packaged ham at 4^{o}C, as compared to bacteria grown in BHIB. Andersen et al. (102) reported that *L. monocytogenes* grown under oxygen-restricted conditions were more invasive to Caco-2 cells and yield higher bacterial loads in organs after oral guinea pig challenge compared to bacteria grown without oxygen-restriction. In contrast to our findings, Olesen et al. (20) reported that *L. monocytogenes* strain O57 *prfA* transcript levels were significantly higher in bacteria grown in BHI compared to in liver pâté at 7^{o}C. Possible explanations for these observed differences include that the *L. monocytogenes* was grown on liver pâtés under aerobic conditions, as well as differences in the length of incubation.

Although PrfA-dependent genes represent *bona-fide* virulence genes (103-105), differential regulation of these genes under the complex conditions of food matrices appears to occur, with oxygen restricting conditions and carbohydrate availability possibly representing environmental cues affecting expression of the PrfA regulon. In addition, a role for PrfA in the survival and proliferation of *L. monocytogenes* outside the human host and on/in specific food matrices cannot be excluded, as supported by data that suggest that PrfA contributes to *L. monocytogenes* biofilm formation (106). Along with previously reported studies, our data suggest that growth

conditions can have multifaceted effects on gene expression and cell physiology, which reach beyond specific adaptations to nutrient availability and stress conditions encountered.

Increasing evidence supports that food related factors that are not easily simulated in laboratory medium likely play important roles in growth and survival of foodborne pathogens in different foods. Use of RNA-seq-based transcriptomic profiling allows for detailed assessment of the physiological state of pathogens present on/in food matrices and provides novel insights in adaptations of foodborne pathogens to the complex conditions on/in specific food matrices and environmental conditions. This type of detailed information will open up a number of new avenues to improve food safety. For example, the type of information presented here could pave the way for developing better detection methods (e.g., methods targeting highly expressed RNA molecules) and may even provide for improved risk assessments that account for the fact that virulence of a given pathogen may be affected considerably by its physiological state, which clearly depends on a number of factors including, but not limited to, food matrix, temperature, and packaging strategies. Most importantly, detailed data on pathogen adaptation to different complex conditions of food matrices may hold the key to development of more efficient control strategies and will move development of control strategies from traditional trial and error approaches to a rational design type approaches for the development of new growth inhibitors.

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CHAPTER 5

CONCLUSIONS

The ability to survive and grow in nutrient limited food processing environments and food products that are stored at low temperatures elevates the risk of food contamination and transmission of *L. monocytogenes* through foods to humans. Our data suggest that, strain variability, clonal cluster variability, genetic lineage, matrix (MBHIB versus CSS), and food packaging method (anaerobic versus aerobic) may significantly influence the growth rate of *L. monocytogenes* in defined media, the efficacy of antimicrobials against *L. monocytogenes,* and the gene expression profile of *L. monocytogenes* during late log phase of growth.

In the first study, we used 10G-MLST, which targets ten housekeeping genes, to investigate the diversity and phylogenetic distribution of *L. monocytogenes* strains with epidemic potential. We also compared the classification result based on 10G-MLST scheme to the ones based on previously described typing schemes (CCs identified by using 7G-MLST scheme and ECs identified by using MvLST), to understand the correlations among them and enable cross reference across the phylogenetic classification results of these schemes. The clonal framework established by using 10G-MLST allowed us to compare the maximum growth rate of *L. monocytogenes* isolates growing in chemically defined media at 16^{o}C by 10G-TLV-CCs (where all STs of the same CC differ from one another in three of the ten alleles). Our findings demonstrated that the 124 *L. monocytogenes* isolates are genetically diverse, and the outbreak-associated isolates were wide-spread through the diversity of *L. monocytogenes.* We also identified that, among the 105 isolates tested, there was considerable variation in maximum growth rate in a nutrient limited growth condition. We found that certain genetic subgroups of

lineage II strains could grow faster than some genetic subgroups of lineage I strains under this nutrient limited condition, which may facilitate their persistence in non-host environments, thus leading to a higher risk of food contamination and transmission of this pathogen through foods to humans.

In the second study, we (i) tested the efficacy of three antimicrobials as well as their binary combinations against *L. monocytogenes* growing on CSS and in MBHIB at 7^{o}C, and (ii) investigated that whether the antimicrobial efficacy data generated from studies in laboratory medium is able to quantitatively predict efficacy in food matrices. Among all antimicrobial treatments, the combination of PL and SDA had synergistic effects and led to the greatest increase in λ both on CSS and in MBHIB. Treatments with NI significantly reduced initial cell density, the combination of NI and PL led to the greatest reductions in N_{max} on CSS, while bactericidal effects of NI on *L. monocytogenes* were highly strain dependent. We observed positive correlations between the growth parameters obtained from MBHIB and CSS, indicating that growth of *L. monocytogenes* in broth, to some extent, qualitatively reflected characteristics of growth on CSS under antimicrobial stresses. Results from MBHIB could quantitatively predict the variability of growth parameters obtained from CSS for lineage II strains, but not for lineage I strains. Our data suggest that (i) strain variability, genetic lineage, and matrix (BHI versus cold-smoked salmon) can significantly affect the efficacy of NI and organic acids against *L. monocytogenes*, and (ii) data from BHI broth may not be able to accurately predict the variability of antimicrobial effects against *L. monocytogenes* on cold smoked salmon.

In the third study, we overcame the technical difficulties associated with isolating high quality bacterial RNA from the seafood matrix, and took advantage of the probe- and annotation-

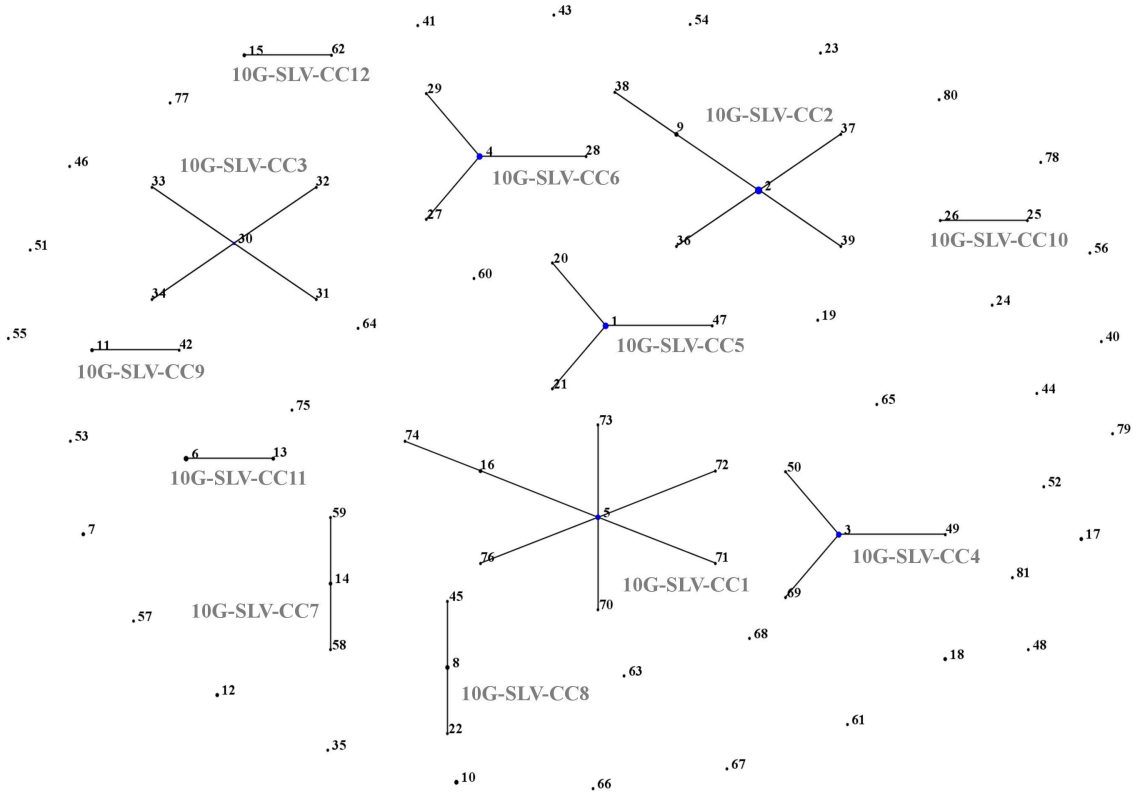
independence of RNA-seq technology to explore the genome-wide gene expression of *L. monocytogenes* grown in a food matrix. We compared the transcriptomes of *L. monocytogenes* strain H7858 grown on CSS and in MBHIB at 7^{o}C, and identified specific transcriptional profiles of *L. monocytogenes* growing on vacuum-packaged CSS. Significant differential transcription of 149 genes was observed (FDR<0.05, fold change ≥ 2.5), and 88 and 61 genes were up- and down regulated in H7858 grown on CSS relative to in MBHIB. Specifically, we found that genes encoding proteins involved in cobalamin biosynthesis as well as ethanolamine and 1,2-propanediol utilization, have significantly higher transcript levels in H7858 grown on CSS compared to in MBHIB. The difference between transcriptional profiles of *L. monocytogenes* growing on CCS and in MBHIB highlighted the importance of the investigation of *L. monocytogenes* transcriptome in a real food matrix. Use of RNA-seq-based transcriptomic profiling allows for detailed assessment of the physiological state of pathogens present in food matrices at an unparalleled resolution and provides novel insights in adaptations of foodborne pathogens to specific food matrices and environmental conditions.

Taken together, our studies in long term may facilitate the surveillance, detection, and control of *L. monocytogenes* strains that are more likely to cause outbreaks and sporadic listeriosis cases. Our data highlighted the importance of using molecular typing methods that are amenable to evolutionary analysis for a further understanding of the phylogenetic distribution of *L. monocytogenes* isolates and for the phenotypic characterization of genetic subgroups of *L. monocytogenes*. Growth parameter data collected in our studies, when combined with genetic and cellular level studies, could provide an improved mechanistic understanding of the cellular response and resistance development of *L. monocytogenes* to different antimicrobials. Detailed transcriptional profile data on pathogen adaptation to different food matrices may provide targets

for development of novel and improved strategies to control *L. monocytogenes* growth on this RTE food.

APPENDIX 1.

SUPPLEMENTAL FIGURES



Supplemental Figure 2.1. The 10G-SLV-CCs and singletons generated by eBURST.

10G-SLV-CCs were identified by using 10G-MLST scheme and eBURST algorithm, where all 10G-STs of the same 10G-SLV-CC differ one another in one of the ten alleles, singletons were accordingly defined as the 10G-STs that differ from all of the other STs in more than one of the ten alleles. The ancestral 10G-ST of a 10G-SLV-CC is represented by a blue dot, and the other 10G-STs are represented by black dot. The size of each dot reflects the number of isolates within a 10G-ST. Only single-locus-variant links are shown within each 10G-SLV-CCs, individual dots represent singletons.

APPENDIX 2.

SUPPLEMENTAL TABLES

Supplemental Table S2.1. Information of *L. monocytogenes* isolates used

Strain ID[a]	Alias	Lineage	Source	Sero-type	Ribo-type	10G-ST[b]	10G-SLV-CC[c]	10G-TLV-CC[d]	7G-ST[e]	7G-CC[f]	EC[g]	μ_{max}[h] [log(OD600/ml)/day]	Genome Accession #	Source of 10G-MLST data
07PF0776		I	human (myocardial abscess), sporadic	4b	NA[i]	42	9	12	4	4	NA	NA	NC_017728.1	(1)
08_5578		II	2008, Canadian deli meat outbreak	1/2a	NA	17	S	S	292	8	V	NA	NC_013766.1	(2)
08_9523		II	2008, Canadian deli meat outbreak	1/2a	NA	17	S	S	120	8	V	NA	NC_013768.1	(2)
ATCC 19117		I	animal (sheep), sporadic	4d	NA	2	2	2	2	2	IV	NA	FR733643	(3)
Finland1998		II	1998, Finland butter outbreak	3a	NA	10	S	S	155	NA	NA	NA	AART00000000	(4)
FSL C1-0051	B98-814	II	1998, human sporadic	NA	1053A	10	S	S	NA	NA	NA	0.054	NA	CUFSL[j]
FSL C1-0111	B98-4374	II	1998, human, sporadic	1/2a	1039E	77	S	1	NA	NA	NA	0.037	NA	CUFSL
FSL C1-0115	2006650	II	1998, human, sporadic	3a	1039C	80	S	S	370	S	NA	0.047	NA	CUFSL
FSL C1-0122	B98-4581	I	1998, human, sporadic	4b	1038B	22	8	8	1	1	NA	0.043	NA	CUFSL
FSL E1-0041	30384/FSL F2-329	I	2000, animal (sheep), clinical	1/2b	1042C	41	S	S	NA	NA	NA	0.051	NA	CUFSL
FSL E1-0154	As-6 48h	II	2001, soil, environment	NA	1053A	65	S	S	NA	NA	NA	0.048	NA	CUFSL
FSL E1-0201	Cs-4ii 24h	I	2001, soil, environment	NA	1051C	4	6	6	NA	NA	NA	0.053	NA	CUFSL
FSL F2-0018	B99-3991	I	1999, human, sporadic	4b	1042B	24	S	9	NA	NA	NA	0.047	NA	CUFSL
FSL F2-0032	99B08871/BAC9900004653	II	1999, food (smoked whitefish)	1/2a	1045B	66	S	S	NA	NA	NA	0.054	NA	CUFSL
FSL F2-0039	B99-4843	II	1999, human, sporadic	1/2a	1030B	63	S	S	NA	NA	NA	0.049	NA	CUFSL
FSL F2-0048	NYC99-119G	II	1999, human, sporadic	1/2a	1053A	70	1	1	NA	NA	NA	0.063	NA	CUFSL
FSL F2-0091	NYC99-141G	I	1999, human, sporadic	4b	1042A	2	2	2	NA	NA	NA	0.055	NA	CUFSL
FSL F2-0141	NYC99-146G	II	1999, human, sporadic	1/2a	1053A	5	1	1	NA	NA	NA	0.055	NA	CUFSL
FSL F2-0194	99190247	II	1999, human, sporadic	NA	1030A	68	S	3	7	7	NA	0.063	NA	CUFSL

Supplemental Table S2.1 (Continued)

FSL F2-0216	99191915	II	1999, human, epidemic	1/2a	1039 A	10	S	S	NA	NA	NA	0.054	NA	CUFSL
FSL F2-0237	99B13372/B00 00000265	II	1999, food (smoked salmon)	1/2a	1062 D	18	S	1	NA	NA	NA	0.045	NA	CUFSL
FSL F2-0270	190144	III	1999, human, sporadic	4a	18007 A	54	S	S	NA	NA	NA	0.054	NA	CUFSL
FSL F2-0366	B00-2431	I	2000, human, sporadic	4b	1042B	26	10	9	NA	NA	NA	0.042	NA	CUFSL
FSL F2-0369	00B04594/BA C0000002476	I	2000, food (RTE pasta salad)	1/2b	1042C	1	5	4	NA	NA	NA	0.043	NA	CUFSL
FSL F2-0405	B00-3127	II	2000, human, sporadic	NA	1053 A	71	1	1	NA	NA	NA	0.054	NA	CUFSL
FSL F2-0493	00B07564/BA C0000004013	I	2000, food (raw chunk beef patties)	1/2b	1024 A	35	S	S	NA	NA	NA	0.047	NA	CUFSL
FSL F2-0515	00B08520/BA C0000004359	II	2000, food (RTE turkey deli meat)	1/2a	1062 A	15	12	13	NA	NA	NA	0.039	NA	CUFSL
FSL F2-0516	B00-4383	II	2000, human, sporadic	1/2a	1053 A	16	1	1	NA	NA	NA	0.02	NA	CUFSL
FSL F2-0521	00B08499/BA C0000004459	I	2000, food (smoked fish salad)	1/2b	1040 A	31	3	5	NA	NA	NA	0.048	NA	CUFSL
FSL F2-0539	EGD-e	II	1924 animal (rabbit)	1/2a	1039C	6	11	10	35	9	NA	0.044	NC_00321 0.1	(5)
FSL F2-0656	NYC01-129g	I	2001, human, sporadic	4b	1042 A	2	2	2	NA	NA	NA	0.041	NA	CUFSL
FSL F2-0661	B01-1825	I	2001, human, sporadic	4b	1042 A	2	2	2	NA	NA	NA	0.041	NA	CUFSL
FSL F2-0693	NYC01-192g	I	2001, human, sporadic	1/2b	1042B	60	S	S	NA	NA	NA	0.036	NA	CUFSL
FSL F2-0699	NYC02-16g	I	2002, human, sporadic	NA	1051 A	14	7	7	NA	NA	NA	0.049	NA	CUFSL
FSL F3-0566	NYC03-216G	II	2003, human, sporadic	NA	1053 A	73	1	1	NA	NA	NA	0.066	NA	CUFSL
FSL F3-0631	3191076	II	2003, human, sporadic	NA	1051B	3	4	3	NA	NA	NA	0.048	NA	CUFSL
FSL F3-0744	M02AE657	II	2002, human, sporadic	NA	1053 A	3	4	3	NA	NA	NA	0.058	NA	CUFSL
FSL F3-0757	M02AE1101	I	2002, human, sporadic	NA	1051 A	14	7	7	NA	NA	NA	0.028	NA	CUFSL
FSL F3-0950	BAC05000059 69	I	2005, human, sporadic	NA	1051C	4	6	6	NA	6	NA	0.049	NA	CUFSL
FSL F3-0995	BAC06000021 27	II	2006, human, sporadic	NA	1053 A	18	S	1	NA	NA	NA	0.055	NA	CUFSL
FSL F6-0084	nyc04-364G	II	2004, human, sporadic	NA	1053 A	3	4	3	NA	NA	NA	0.055	NA	CUFSL

Supplemental Table S2.1 (Continued)

FSL F6-0095	nyc05-199G	I	2005, human, sporadic	NA	1051C	28	6	6	NA	NA	NA	0.055	NA	CUFSL
FSL F6-0154	J2818	II	2000, food, US turkey deli meat outbreak	1/2a	1053 A	5	1	1	86	11	III	0.053	AARX000 00000	CUFSL
FSL F6-0366	H7858	I	1998-1999, US, hot dog outbreak	4b	1044 A	4	6	6	6	6	II	0.034	AADR000 00000	(6)
FSL F6-0386	07B05187A-1	I	2007, food (smoked salmon)	NA	1051B	20	5	4	NA	NA	NA	0.049	NA	CUFSL
FSL G2-0003	LO28	II	human, reference strain	1/2c	1056 A	61	S	10	210	9	NA	0.034	AARY000 00000	CUFSL
FSL H5-0804	06B00640D-1	I	2006, environment (deli floor drain)	1/2b	1051 A	59	7	7	NA	NA	NA	0.052	NA	CUFSL
FSL J1-0020	DD6609	I	1987, human, Philadelphia multiple foods outbreak	4b	1042	9	2	2	NA	2	NA	0.052	NA	CUFSL
FSL J1-0022	DD942/NCTC 4885	II	human sporadic	1/2c	1039C	13	11	10	NA	9	NA	0.052	NA	CUFSL
FSL J1-0049	HO462	I	human sporadic	3c	1042C	1	5	4	3	3	NA	0.028	NA	CUFSL
FSL J1-0101	F6900/G3975/ DD6292	II	1998, human, sporadic	1/2a	1053 A	5	1	1	86	11	III	0.04	AARU000 00000.2	(7)
FSL J1-0108	L4738/TS27/D D6304	I	1981, human, Halifax coleslaw outbreak	4b	1038B	8	8	8	1	1	NA	0.059	NA	CUFSL
FSL J1-0110	F2365	I	1985, food, Mexican-style cheese outbreak	4b	1038B	8	8	8	1	1	I	0.052	NC_00297 3.6	CUFSL
FSL J1-0116	TS38/L3306/D D6315	I	1987-1989, human, UK and Ireland Pâté outbreak	4b	1042B	2	2	2	2	2	NA	0.062	NA	CUFSL
FSL J1-0125	TS57/L4706/D D6334	II	human, sporadic	1/2c	1039C	6	11	10	NA	9	NA	0.039	NA	CUFSL
FSL J1-0126	TS60/L4486b/ DD6337	I	1983-1987, human, Switzerland cheese outbreak	4b	1038B	8	8	8	1	1	NA	0.033	NA	CUFSL
FSL J1-0129	TS65/L3238/D D6342	I	1987-1989, human, UK and Ireland Pâté outbreak	4bx	1042B	2	2	2	NA	2	NA	0.046	NA	CUFSL
FSL J1-0169	B96-1303	I	1996, human, sporadic	3b	1052 A	7	S	S	5	5	NA	0.051	NA	CUFSL

Supplemental Table S2.1 (Continued)

FSL J1-0175	B97-471	I	environment (water)	1/2b	1042 A	58	7	7	87	S	NA	0.052	AARK000 00000	CUFSL
FSL J1-0194	B97-904	I	1997, human, sporadic	1/2b	1042B	30	3	5	88	S	NA	0.036	AARJ000 00000	CUFSL
FSL J1-0208	98-209	III	1998, animal (caprine), sporadic	4a	10142	55	S	S	569	NA	NA	NA	AARL000 00000	(den Bakker et al. 2012)
FSL J1-0220	C7942	I	1979, human, vegetable outbreak	4b	1042B	2	2	2	NA	NA	NA	0.047	NA	CUFSL
FSL J1-0225	Scott A	I	1983, human, United States outbreak	4b	1042B	9	2	2	290	2	IV	0.058	CM00115 9.1	CUFSL
FSL J2-0003	DL 696073-1A	II	1993, animal (bovine), clinical	1/2a	1039C	79	S	S	89	NA	NA	0.048	AARM00 000000	CUFSL
FSL J2-0045	DL 662205-1A	I	1992, animal (sheep), clinical	4b	1042B	25	10	9	NA	NA	NA	0.044	NA	CUFSL
FSL J2-0054	CU-BR 1/93	II	1993, animal (sheep), clinical	1/2a	1045B	48	S	S	412	S	NA	0.061	NA	CUFSL
FSL J2-0071	DL 758453-1A	III	1994, animal (bovine), clinical	4c	1061 A	52	S	11	131	71	NA	NA	AARN000 00000	(4)
FSL L3-0051	A1-F1-021402	I	2002,food (RTE salmon)	1/2b	1042C	1	5	4	NA	3	NA	0.034	NA	CUFSL
FSL L3-0123	A2-E3-030502	II	2002, environment (floor drain)	NA	1053 A	72	1	1	NA	NA	NA	0.061	NA	CUFSL
FSL L4-0096	B1-E1-100702	II	2002, environment (fish processing plant drain)	NA	1051B	50	4	3	NA	NA	NA	0.059	NA	CUFSL
FSL L4-0100	B1-E13-100702	II	2002, environment (fish processing floor mat)	NA	1051B	49	4	3	NA	NA	NA	0.059	NA	CUFSL
FSL L4-0151	A2-E16-101502	II	2002, environment (raw salmon room floor drain)	1/2a	1062 A	15	12	13	NA	NA	NA	0.044	NA	CUFSL
FSL L4-0396	A1-E1-121602	II	2002, environment (finished product area drain)	1/2a	1039C	67	S	S	NA	NA	NA	0.048	NA	CUFSL
FSL M1-0006	B98-506	I	1998, human, sporadic	NA	1051B	19	S[i]	4	NA	NA	NA	0.012	NA	CUFSL
FSL M2-0042	B99-1257	I	1999, human, sporadic	4b	1042B	37	2	2	NA	NA	NA	0.041	NA	CUFSL

Supplemental Table S2.1 (Continued)

FSL N1-0017	1B-22-1	I	1998, food	1/2b	1042C	21	5	4	3	3	VIII	0.033	AARP00000	CUFSL
FSL N1-0061	2D-31-2	I	1998, food (salmon brine)	4b	1044 A	27	6	6	NA	NA	NA	0.053	NA	CUFSL
FSL N1-0225	H7550	I	1998-1999, human, US hot dog outbreak	4b	1044 A	29	6	6	6	6	NA	0.05	NA	CUFSL
FSL N1-0260	NYC98-150	I	human	1	1051 A	33	3	5	NA	NA	NA	0.031	NA	CUFSL
FSL N3-0165	LS2B	II	2002, environment (soil)	1/2a	1045 A	78	S	S	90	90	NA	0.057	AARQ00000	CUFSL
FSL N3-0692	TS2B	I	2002, environment (soil)	4b	1051C	4	6	6	6	6	NA	0.054	NA	CUFSL
FSL N3-0780	TF2C	I	2002, animal (bovine feces)	4b	1051C	4	6	6	6	6	NA	0.055	NA	CUFSL
FSL R2-0011	N-7371	II	2000, food (RTE deli salad)	2	1053 A	16	1	1	NA	NA	NA	0.057	NA	CUFSL
FSL R2-0089	N-7432	II	2000, food (RTE deli salad)	NA	1062 A	62	12	13	NA	NA	NA	0.047	NA	CUFSL
FSL R2-0154	N-7494	I	2001, food (smoked seafood)	1/2b	1042C	1	5	4	NA	3	NA	0.055	NA	CUFSL
FSL R2-0182	N-7522	I	2001, food (smoked seafood)	1/2b	1043 A	7	S	S	NA	5	NA	0.061	NA	CUFSL
FSL R2-0487	N-7817	II	2001, food (RTE bagged salad)	NA	1053 A	74	1	1	NA	NA	NA	0.059	NA	CUFSL
FSL R2-0493	N-7823	II	2001, food (RTE deli salad)	NA	1056 A	64	S	S	NA	NA	NA	0.018	NA	CUFSL
FSL R2-0499	FSL R2-0603/J0161	II	2000, human, US turkey deli meat outbreak	1/2a	1053 A	76	1	1	11	11	III	0.059	AARW00000000.2	(7)
FSL R2-0501	J0211	I	2000, human, cheese outbreak	4b	1042B	40	S	S	558	S	NA	0.043	NA	CUFSL
FSL R2-0502	G6003	I	1994, food, Illinois chocolate milk outbreak	1/2b	1051B	1	5	4	3	3	NA	0.04	NA	CUFSL
FSL R2-0557	G3963	I	human, sporadic	1/2c	1042B	34	3	5	NA	NA	NA	0.033	NA	CUFSL
FSL R2-0559	F6854/G3965	II	1998, food, sporadic	1/2a	1053 A	5	1	1	11	11	III	0.054	AADQ00000	(6)
FSL R2-0561	G3969	II	human, sporadic	1/2c	1039C	6	11	10	9	9	NA	0.053	AARS00000	CUFSL

Supplemental Table S2.1 (Continued)

FSL R2-0578	G3996	I	1983, human, Boston milk outbreak	4b	1042B	38	2	2	NA	2	NA	0.05	NA	CUFSL
FSL R2-0583	G4003	I	1983, human, Boston milk outbreak	4b	1042B	9	2	2	NA	2	NA	0.039	NA	CUFSL
FSL R2-0585	L3350	I	1987-1989, food, UK and Ireland Pâté outbreak	4b	1042B	2	2	2	NA	2	NA	0.021	NA	CUFSL
FSL R2-0589	G4012/L3334	I	1987-1989, food, UK and Ireland Pâté outbreak	4b	1042B	36	2	2	NA	2	NA	0.049	NA	CUFSL
FSL R2-0598	G6055	I	1994, human, Illinois chocolate milk outbreak	1/2b	1051B	1	5	4	NA	3	NA	0.06	NA	CUFSL
FSL R2-0763	J1735	I	2002, human, Northeastern states sliced deli meat outbreak	4b	1044 A	4	6	6	6	6	NA	0.044	NA	CUFSL
FSL R6-0896		II	2007, environment (floor drain)	NA	1053 A	3	4	3	NA	7	NA	0.064	NA	CUFSL
FSL R8-0879		II	2008, environment (floor drain)	1/2a	1053 A	75	S	1	NA	NA	NA	0.061	NA	CUFSL
FSL R8-5459		I	2010, environment (floor)	NA	1040 A	32	3	5	NA	NA	NA	0.05	NA	CUFSL
FSL S10-630		I	2010, environment (produce farm)	NA	1044B	43	S	S	NA	NA	NA	0.048	NA	CUFSL
FSL S4-0440	U7-20-1	I	2002, environment (sidewalk floor)	1/2b	1042B	23	S	S	379	NA	NA	0.049	NA	CUFSL
FSL S4-0643	U9-14-3	I	2002, environment (bench)	4b	1044B	11	9	12	NA	4	NA	0.052	NA	CUFSL
FSL S4-0848	U13-31-2	I	2002, environment (sidewalk floor)	4b	1042B	39	2	2	2	2	NA	0.034	NA	CUFSL
FSL T1-0073	A2-R4-032601	II	2001, food (raw norweigan)	1/2a	1023C	81	S	1	NA	NA	NA	0.065	NA	CUFSL
FSL X1-0001	10403S	II	1987, human, sporadic	1/2a	1030 A	69	4	3	85	7	VII	0.047	AARZ000 00000	CUFSL
FSL X1-0002	L99	III	1950, food (cheese)	4a	NA	12	S	S	201	NA	NA	NA	FM211688	(3)
HCC23		III	animal (healthy channel catfish)	4a	NA	12	S	S	201	NA	NA	NA	NC_01166 0.1	(8)

Supplemental Table S2.1 (Continued)

HPB2262	I	1997, Italian gastroenteritis outbreak	4b	NA	2	2	2	2	2	IV	NA	AATL00000000	(4)
L312	I	food (cheese)	4b	NA	11	9	12	4	4	NA	NA	FR733642	(3)
SLCC 2372	II	1935, human, sporadic	1/2c	NA	13	11	10	122	9	NA	NA	FR733648	(3)
SLCC 2376	III	poultry	4c	NA	44	S	11	71	71	NA	NA	FR733651	(3)
SLCC 2378	I	poultry	4e	NA	45	8	8	73	1	I	NA	FR733644	(3)
SLCC 2479	II	1966, human, sporadic	3c	NA	6	11	10	9	9	NA	NA	FR733649	(3)
SLCC 2540	I	1956, human, sporadic	3b	NA	46	S	S	617	3	NA	NA	FR733645	(3)
SLCC 2755	I	1967, animal (chinchilla)	1/2b	NA	47	5	4	66	3	VIII	NA	FR733646	(3)
SLCC 5850	II	1924, animal (rabbit)	1/2a	NA	3	4	3	12	7	VII	NA	FR733647	(3)
SLCC 7179	II	1986, food (cheese)	3a	NA	51	S	S	91	NA	NA	NA	FR733650	(3)
W1-110	III	Unknown	4c	1055 A	53	S	S	NA	NA	NA	0.06	NA	CUFSL
W1-111	III	Unknown	4c	18036	56	S	S	NA	NA	NA	0.05	NA	CUFSL
W1-112	III	Unknown	4a	1033 A	57	S	S	NA	NA	NA	0.052	NA	CUFSL

[a]Isolates are listed in alphabetical order of the strain IDs.
[b]10G-ST, sequence type (ST) identified by using 10-gene multilocus sequence typing (10G-MLST) scheme.
[c]10G-SLV-CC, single-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the clonal complex (CC) differ from one another in one of the ten alleles.
[d]10G-TLV-CC, triple-locus-variant-clonal complex identified by using 10G-MLST scheme and eBURST algorithm, where all STs in the same CC differ from one another in three of the ten alleles.
[e]7G-ST, ST identified by using 7-gene multilocus sequence typing (7G-MLST) scheme.
[f]7G-CC, CC identified by using 7G-MLST scheme, where all STs in the same CC differ from one another in one of the seven alleles.
[g]EC, epidemic clone
[h]μmax, growth parameter maximum growth rate of *L. monocytogenes* isolates growing in defined medium (DM) at 16oC.
[i]NA, not available.
[j]CUFSL, Cornell University Food Safety Lab.
[k]S, singleton, (i) for 10G-SLV-CCs and 10G-TLV-CCs, singletons were defined as STs differing from all of the other STs in more than one or more than three of the ten alleles, respectively, (ii) for 7G-CCs, singletons were defined as STs differing from all of the other STs in more than one of the seven allele

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Supplemental Table S4.1. Upregulated genes in H7858 grown on CCS as compared to it grown in MBHIB at 7°C

H7858 gene name	Gene product	FC[a]
LMOh7858_0048	Putrescine carbamoyltransferase (EC 2.1.3.6)	31.36
LMOh7858_0049	Agmatine/putrescine antiporter, associated with agmatine catabolism	19.56
LMOh7858_0050	Agmatine deiminase (EC 3.5.3.12)	13.67
LMOh7858_0051	Carbamate kinase (EC 2.7.2.2)	18.29
LMOh7858_0176	Zinc ABC transporter, periplasmic-binding protein ZnuA	7.64
LMOh7858_0221	Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	2.83
LMOh7858_0222	Thiol-activated cytolysin	2.54
LMOh7858_0224	Actin-assembly inducing protein ActA precursor	2.86
LMOh7858_0225	Broad-substrate range phospholipase C (EC 3.1.4.3)	3.19
LMOh7858_0311	Multiple sugar ABC transporter, ATP-binding protein	2.54
LMOh7858_0382	Transketolase (EC 2.2.1.1)	3.39
LMOh7858_0383	Transaldolase (EC 2.2.1.2)	3.98
LMOh7858_0384	oxidoreductase, short-chain dehydrogenase/reductase family	3.69
LMOh7858_0385	Ribose 5-phosphate isomerase B (EC 5.3.1.6)	5.86
LMOh7858_0396	Fumarate reductase flavoprotein subunit (EC 1.3.99.1)	6.38
LMOh7858_0499	Internalin B (GW modules)	2.71
LMOh7858_0536	antigen, putative	17.50
LMOh7858_0755	Flagellin protein FlaA	3.43
LMOh7858_0840	FMN-dependent NADH-azoreductase	2.73
LMOh7858_0957	Sulfate permease	3.95
LMOh7858_1214	Cob(III)alamin reductase	3.81
LMOh7858_1215	Propanediol utilization polyhedral body protein PduT	3.72
LMOh7858_1216	Propanediol utilization polyhedral body protein PduU	4.48

Supplemental Table S4.1 (Continued)

LMOh7858_1217	Propanediol utilization protein PduV	6.57
LMOh7858_1217.1	Predicted alpha-ribazole-5-phosphate synthase CblS for cobalamin biosynthesis	6.36
LMOh7858_1219	Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	5.02
LMOh7858_1220	Cobalamin synthase	6.08
LMOh7858_1221	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73)	6.78
LMOh7858_1242	L-threonine 3-O-phosphate decarboxylase (EC 4.1.1.81)	2.77
LMOh7858_1243	Threonine kinase in B12 biosynthesis	2.88
LMOh7858_1245	Alcohol dehydrogenase (EC 1.1.1.1)	4.97
LMOh7858_1249	Ethanolamine ammonia-lyase heavy chain (EC 4.3.1.7)	4.97
LMOh7858_1250	Ethanolamine ammonia-lyase light chain (EC 4.3.1.7)	5.50
LMOh7858_1253	Acetaldehyde dehydrogenase, ethanolamine utilization cluster	5.26
LMOh7858_1254	Ethanolamine utilization protein similar to PduA/PduJ	5.56
LMOh7858_1254.2	Ethanolamine utilization protein similar to PduL	5.89
LMOh7858_1258.1	Ethanolamine utilization protein similar to PduT	6.19
LMOh7858_1260	Ethanolamine permease	5.90
LMOh7858_1262.1	Substrate-specific component CblT of predicted B12-regulated ECF transporter	8.33
LMOh7858_1264	Cobyrinic acid A,C-diamide synthase	10.02
LMOh7858_1265	Adenosylcobinamide-phosphate synthase	12.88
LMOh7858_1266	Cobalt-precorrin-8x methylmutase (EC 5.4.1.2)	11.84
LMOh7858_1267	Cobalt-precorrin-6 synthase, anaerobic	11.27
LMOh7858_1268	Cobalt-precorrin-6y C5-methyltransferase (EC 2.1.1.-)	12.39
LMOh7858_1269	Cobalt-precorrin-6y C15-methyltransferase [decarboxylating] (EC 2.1.1.-)	11.32
LMOh7858_1270	Cobalt-precorrin-4 C11-methyltransferase (EC 2.1.1.133)	9.70

Supplemental Table S4.1 (Continued)

LMOh7858_1271	Cobalamin biosynthesis protein CbiG	11.54
LMOh7858_1272	Cobalt-precorrin-3b C17-methyltransferase	10.29
LMOh7858_1273	Cobalt-precorrin-6x reductase (EC 1.3.1.54)	10.85
LMOh7858_1274	Uroporphyrinogen-III methyltransferase (EC 2.1.1.107)	12.55
LMOh7858_1275	Sirohydrochlorin cobaltochelatase CbiK (EC 4.99.1.3)	10.40
LMOh7858_1276	Cobalt-precorrin-2 C20-methyltransferase (EC 2.1.1.130)	11.76
LMOh7858_1277	Substrate-specific component CbiM of cobalt ECF transporter	9.95
LMOh7858_1278	Additional substrate-specific component CbiN of cobalt ECF transporter	9.02
LMOh7858_1279	Transmembrane component CbiQ of energizing module of cobalt ECF transporter	7.54
LMOh7858_1280	ATPase component CbiO of energizing module of cobalt ECF transporter	5.30
LMOh7858_1281	Cobyric acid synthase	2.94
LMOh7858_1282	Cob(I)alamin adenosyltransferase PduO (EC 2.5.1.17)	2.66
LMOh7858_1329.1	FIG00774262: hypothetical protein	7.76
LMOh7858_1331	transporter	8.12
LMOh7858_2119	PTS system, mannose-specific IIA component (EC 2.7.1.69)	6.31
LMOh7858_2119.1	Putative glucosamine-fructose-6-phosphate aminotransferase	3.42
LMOh7858_2121	putative glucosamine-fructose-6-phosphate aminotransferase	3.99
LMOh7858_2122	PTS system, mannose-specific IID component (EC 2.7.1.69)	5.61
LMOh7858_2123	PTS system, mannose-specific IIC component (EC 2.7.1.69)	4.78
LMOh7858_2124	PTS system, mannose-specific IIB component (EC 2.7.1.69)	4.71
LMOh7858_2253	Maltose phosphorylase (EC 2.4.1.8)	3.45
LMOh7858_2253.1	Maltodextrose utilization protein MalA	5.71
LMOh7858_2255	Maltose/maltodextrin ABC transporter, permease protein MalG	6.32

Supplemental Table S4.1 (Continued)

LMOh7858_2256	Maltose/maltodextrin ABC transporter, permease protein MalF	7.96
LMOh7858_2257	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein	5.74
LMOh7858_2292.2	FIG00774368: hypothetical protein	5.05
LMOh7858_2292.3	Inosose dehydratase (EC 4.2.1.44)	4.27
LMOh7858_2426.4	FIG00775213: hypothetical protein	2.85
LMOh7858_2503	FIG00774521: hypothetical protein	4.96
LMOh7858_2504	Rrf2 family transcriptional regulator	4.24
LMOh7858_2903	serine/threonine protein phosphatase family protein	2.78
LMOh7858_2922	Transketolase (EC 2.2.1.1)	4.77
LMOh7858_2925	Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.251)	7.62
LMOh7858_2926	Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.251)	5.48
LMOh7858_2927	PTS system, galactitol-specific IIC component (EC 2.7.1.69)	4.38
LMOh7858_2928	PTS system, galactitol-specific IIB component (EC 2.7.1.69)	3.17
LMOh7858_2994	Haloacid dehalogenase-like hydrolase	5.34
LMOh7858_2995	Transcription regulator, RpiR family	3.11
LMOh7858_3062	hydrolase, haloacid dehalogenase-like family	7.97
LMOh7858_3063	PTS system, mannitol-specific IIB component (EC 2.7.1.69)	19.28
LMOh7858_3064	oxidoreductase, Gfo/Idh/MocA family	9.36
LMOh7858_3065	N-acetylmannosamine-6-phosphate 2-epimerase (EC 5.1.3.9)	13.04

[a]FC, fold change. FC= average normalized RNA-seq coverage (NRC) (CCS) ÷ average NRC (MBHIB)

Supplemental Table S4.2. Down regulated genes H7858 grown on CCS as compared to it grown in MBHIB at 7°C

H7858 Gene Name	Gene Product	FC[a]
LMOh7858_0107	oxidoreductase, aldo/keto reductase family	0.37
LMOh7858_0175	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein O	0.38
LMOh7858_0246	Dihydropteroate synthase (EC 2.5.1.15)	0.16
LMOh7858_0367	Internalin-like protein (LPXTG motif) Lmo0331 homolog	0.40
LMOh7858_0506	Cell division protein FtsI [Peptidoglycan synthetase] (EC 2.4.1.129)	0.32
LMOh7858_0508	Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfa	0.31
LMOh7858_0625	Imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19)	0.31
LMOh7858_0655	O-acetylhomoserine sulfhydrylase (EC 2.5.1.49) / O-succinylhomoserine sulfhydrylase	0.37
LMOh7858_0667	Transcriptional regulator, MarR family	0.38
LMOh7858_0852.2	GTP pyrophosphokinase (EC 2.7.6.5)	0.32
LMOh7858_0878	acetyltransferase, GNAT family	0.36
LMOh7858_0940	Putative peptidoglycan bound protein (LPXTG motif) Lmo0880 homolog	0.30
LMOh7858_1015.1	FIG00774399: hypothetical protein	0.27
LMOh7858_1015.2	FIG00774456: hypothetical protein	0.39
LMOh7858_1066.1	FIG00774146: hypothetical protein	0.28
LMOh7858_1202.1	FIG00774448: hypothetical protein	0.00
LMOh7858_1289	N-acetylmuramoyl-L-alanine amidase, family 4	0.39
LMOh7858_1299	Transcriptional regulator, MarR family	0.38
LMOh7858_1308.2	FIG00774960: hypothetical protein	0.24
LMOh7858_1321.2	DUF1801 domain-containing protein	0.36
LMOh7858_1321.3	PhnB protein; putative DNA binding 3-demethylubiquinone-9 3-methyltransfer	0.28

Supplemental Table S4.2 (Continued)

LMOh7858_1339	Trehalose-6-phosphate hydrolase (EC 3.2.1.93)	0.18
LMOh7858_1340	PTS system, trehalose-specific IIB component (EC 2.7.1.69) / PTS system,	0.19
LMOh7858_1511	FIG00774487: hypothetical protein	0.30
LMOh7858_1614	Iron-sulfur cluster regulator IscR	0.38
LMOh7858_1812.1	Hypothetical protein SAV1869	0.38
LMOh7858_1858	Glutamate synthase [NADPH] small chain (EC 1.4.1.13)	0.30
LMOh7858_1859	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)	0.29
LMOh7858_1956	Orotate phosphoribosyltransferase (EC 2.4.2.10)	0.26
LMOh7858_1957	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)	0.27
LMOh7858_1958	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)	0.29
LMOh7858_1959	Dihydroorotate dehydrogenase electron transfer subunit (EC 1.3.3.1)	0.22
LMOh7858_1960	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	0.21
LMOh7858_1961	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	0.22
LMOh7858_1965.1	FIG00774989: hypothetical protein	0.37
LMOh7858_1977	Copper chaperone	0.38
LMOh7858_2167	Cell division protein FtsL	0.37
LMOh7858_2179	Excinuclease ABC subunit A paralog of unknown function	0.38
LMOh7858_2262.2	FIG00774113: hypothetical protein	0.37
LMOh7858_2337	N-acetylmuramoyl-L-alanine amidase, family 4	0.31
LMOh7858_2343.1	FIG00774091: hypothetical protein	0.18
LMOh7858_2490	L-Cystine ABC transporter, permease protein TcyM	0.18
LMOh7858_2491	L-Cystine ABC transporter, permease protein TcyL	0.12
LMOh7858_2492	L-Cystine ABC transporter, periplasmic cystine-binding protein TcyK	0.31
LMOh7858_2495	HTH-type transcriptional regulator YtlI, LysR family	0.26

Supplemental Table S4.2 (Continued)

LMOh7858_2521	PTS system, IIB component	0.38
LMOh7858_2566.1	FIG00774101: hypothetical protein	0.23
LMOh7858_2584.1	FIG00774650: hypothetical protein	0.40
LMOh7858_2635	FIG00774998: hypothetical protein	0.25
LMOh7858_2635.1	Hypothetical protein, homolog of fig|393130.3.peg.2627	0.20
LMOh7858_2717.1	FIG00774295: hypothetical protein	0.13
LMOh7858_2717.2	FIG00774092: hypothetical protein	0.19
LMOh7858_2752	lipoprotein, putative	0.30
LMOh7858_2951	Mg(2+) transport ATPase, P-type (EC 3.6.3.2)	0.27
LMOh7858_3026.1	hypothetical protein	0.31
LMOh7858_3027	Beta-glucosidase (EC 3.2.1.21); 6-phospho-beta-glucosidase (EC 3.2.1.86)	0.24
LMOh7858_3028	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	0.12
LMOh7858_3066.6	hypothetical secreted protein	0.30
LMOh7858_3076	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	0.24
LMOh7858_3082	transporter	0.27
LMOh7858_3083	Catalyzes the cleavage of p-aminobenzoyl-glutamate to p-aminobenzoate and glutamate	0.29

[a]FC, fold change. FC= average normalized RNA-seq coverage (NRC) (CCS) ÷ average NRC (MBHIB)

Supplemental Table S4.3. Genes involved in cobalamin biosynthesis upregulated in H7858 grown on CSS compared to in MBHIB at 7°C

Gene name in			H7858 gene product	FC[b]
H7858	EGD-e [a]	10403S [a]		
LMOh7858_1214	lmo1142	LMRG_00585	Cob(III)alamin reductase	3.81
LMOh7858_1217.1	lmo1146	LMRG_00589	Predicted alpha-ribazole-5-phosphate synthase CblS for cobalamin biosynthesis	6.36
LMOh7858_1219	lmo1147	LMRG_00590	Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	5.02
LMOh7858_1220	lmo1148 (*cobS*)	LMRG_00591	Cobalamin synthase	6.08
LMOh7858_1221	lmo1149	LMRG_00592	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73)	6.78
LMOh7858_1242	lmo1169 (*cobD*)	LMRG_00612	L-threonine 3-O-phosphate decarboxylase (EC 4.1.1.81)	2.77
LMOh7858_1262.1	lmo1190	LMRG_00636	Substrate-specific component CblT of predicted B12-regulated ECF transporter	8.33
LMOh7858_1264	lmo1191 (*cobB*)	LMRG_00637	Cobyrinic acid A,C-diamide synthase	10.02
LMOh7858_1265	lmo1192 (*cobD*)	LMRG_00638	Adenosylcobinamide-phosphate synthase	12.88
LMOh7858_1266	lmo1193	LMRG_00639	Cobalt-precorrin-8x methylmutase (EC 5.4.1.2)	11.84
LMOh7858_1267	lmo1194 *(cbiD*)	LMRG_00640	Cobalt-precorrin-6 synthase, anaerobic	11.27
LMOh7858_1268	lmo1195 (*cbiE*)	LMRG_00641	Cobalt-precorrin-6y C5-methyltransferase (EC 2.1.1.-)	12.39
LMOh7858_1269	lmo1196	LMRG_00642	Cobalt-precorrin-6y C15-methyltransferase [decarboxylating] (EC 2.1.1.-)	11.32
LMOh7858_1270	lmo1197 *(cbiF*)	LMRG_00643	Cobalt-precorrin-4 C11-methyltransferase (EC 2.1.1.133)	9.70
LMOh7858_1271	lmo1198 (*cbiG*)	LMRG_00644	Cobalamin biosynthesis protein CbiG	11.54
LMOh7858_1272	lmo1199 (*cbiH*)	LMRG_00645	Cobalt-precorrin-3b C17-methyltransferase	10.29

Supplemental Table S4.3 (Continued)

LMOh7858_1273	lmo1200	LMRG_00646	Cobalt-precorrin-6x reductase (EC 1.3.1.54)	10.85
LMOh7858_1274	lmo1201	LMRG_00647	Uroporphyrinogen-III methyltransferase (EC 2.1.1.107) / Uroporphyrinogen	12.55
LMOh7858_1275	lmo1202 (*cbiK*)	LMRG_00648	Sirohydrochlorin cobaltochelatase CbiK (EC 4.99.1.3)	10.40
LMOh7858_1276	lmo1203 (*cbiL*)	LMRG_00649	Cobalt-precorrin-2 C20-methyltransferase (EC 2.1.1.130)	11.76
LMOh7858_1277	lmo1204	LMRG_00650	Substrate-specific component CbiM of cobalt ECF transporter	9.95
LMOh7858_1278	lmo1205	LMRG_00651	Additional substrate-specific component CbiN of cobalt ECF transporter	9.02
LMOh7858_1279	lmo1206 (*cbiQ*)	LMRG_00652	Transmembrane component CbiQ of energizing module of cobalt ECF transporter	7.54
LMOh7858_1280	lmo1207	LMRG_00653	ATPase component CbiO of energizing module of cobalt ECF transporter	5.30
LMOh7858_1281	lmo1208 (*cobQ*)	LMRG_00654	Cobyric acid synthase	2.94
LMOh7858_1282	lmo1209	LMRG_00655	Cob(I)alamin adenosyltransferase PduO (EC 2.5.1.17)	2.66

[a]Strain EGD-e (GenBank accession no.: NC_003210) and10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;
[b]FC, fold change, FC= average NRC(CSS) ÷ average NRC(MBHIB); only genes with FDR <0.05 are listed.

Supplemental Table S4.4. Upregulated genes involved in ethanolamine and 1,2-propanediol utilization in H7858 grown on CSS compared to in MBHIB at 7°C

Gene name in			H7858 gene product	FC[b]
H7858	EGD-e[a]	10403S[a]		
Ethanolamine utilization				
LMOh7858_1245	lmo1171 *(pduQ)*[c]	LMRG_00617	Alcohol dehydrogenase (EC 1.1.1.1)	4.97
LMOh7858_1249	lmo1175 *(eutB)*	LMRG_00621	Ethanolamine ammonia-lyase heavy chain (EC 4.3.1.7)	4.97
LMOh7858_1250	lmo1176 *(eutC)*	LMRG_00622	Ethanolamine ammonia-lyase light chain (EC 4.3.1.7)	5.50
LMOh7858_1253	lmo1179	LMRG_00625	Acetaldehyde dehydrogenase, ethanolamine utilization cluster	5.26
LMOh7858_1254	lmo1180	LMRG_00626	Ethanolamine utilization protein similar to PduA/PduJ	5.56
LMOh7858_1254.2	lmo1182	LMRG_00628	Ethanolamine utilization protein similar to PduL	5.89
LMOh7858_1258.1	lmo1185	LMRG_00631	Ethanolamine utilization protein similar to PduT	6.19
LMOh7858_1260	lmo1186	LMRG_00632	Ethanolamine permease	5.90
1,2-propanediol utilization				
LMOh7858_1214	lmo1142	LMRG_00585	Cob(III)alamin reductase	3.81
LMOh7858_1215	lmo1143	LMRG_00586	Propanediol utilization polyhedral body protein PduT	3.72
LMOh7858_1216	lmo1144	LMRG_00587	Propanediol utilization polyhedral body protein PduU	4.48
LMOh7858_1217	lmo1145	LMRG_00588	Propanediol utilization protein PduV	6.57
LMOh7858_1243	lmo1170	LMRG_00613	Threonine kinase in B12 biosynthesis	2.88
LMOh7858_1245	lmo1171 (*pduQ*)[c]	LMRG_00617	Alcohol dehydrogenase (EC 1.1.1.1)	4.97
LMOh7858_1282	lmo1209	LMRG_00655	Cob(I)alamin adenosyltransferase PduO (EC 2.5.1.17)	2.66

[a]Strain EGD-e (GenBank accession no.: NC_003210) and 10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;

[b]FC, fold change, FC= average NRC(CSS) ÷ average NRC(MBHIB); only genes with FDR <0.05 are listed;
[c]As the gene product Alcohol dehydrogenase of the gene LMOh7858_1245 may participate in both of the ethanolamine utilization and the 1,2-propanediol utilization pathways, this gene was listed under both categories.

Supplemental Table S4.5. Upregulated genes involved in carbohydrate transport and utilization in H7858 grown on CSS compared to in MBHIB at 7°C

Gene name in			H7858 gene product	FC[b]
H7858	EGD-e[a]	10403S[a]		
Mannose				
LMOh7858_2119	lmo1997	LMRG_01145	PTS system, mannose-specific IIA component (EC 2.7.1.69)	6.31
LMOh7858_2122	lmo2000	LMRG_01148	PTS system, mannose-specific IID component (EC 2.7.1.69)	5.61
LMOh7858_2123	lmo2001	LMRG_01149	PTS system, mannose-specific IIC component (EC 2.7.1.69)	4.78
LMOh7858_2124	lmo2002	LMRG_01150	PTS system, mannose-specific IIB component (EC 2.7.1.69)	4.71
Galactitol				
LMOh7858_2925	lmo2663	LMRG_02208	Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.251)	7.62
LMOh7858_2926	lmo2664	LMRG_02209	Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.251)	5.48
LMOh7858_2927	lmo2665	LMRG_02210	PTS system, galactitol-specific IIC component (EC 2.7.1.69)	4.38
LMOh7858_2928	lmo2666	LMRG_02211	PTS system, galactitol-specific IIB component (EC 2.7.1.69)	3.17
Mannitol				
LMOh7858_3063	lmo2799	LMRG_01898	PTS system, mannitol-specific IIB component (EC 2.7.1.69)	19.28
Maltose				
LMOh7858_2253	lmo2121	LMRG_01275	Maltose phosphorylase (EC 2.4.1.8)	3.45
LMOh7858_2253.1	lmo2122	LMRG_01276	Maltodextrose utilization protein MalA	5.71
LMOh7858_2255	lmo2123	LMRG_01277	Maltose/maltodextrin ABC transporter, permease protein MalG	6.32
LMOh7858_2256	lmo2124	LMRG_01278	Maltose/maltodextrin ABC transporter, permease protein MalF	7.96

Supplemental Table S4.5 (Continued)

LMOh7858_2257	lmo2125	LMRG_01279	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein	5.74
Pentose phosphate pathway				
LMOh7858_2922	lmo2660	LMRG_02205	Transketolase (EC 2.2.1.1)	4.77
LMOh7858_0382	lmo0342	LMRG_00033	Transketolase (EC 2.2.1.1)	3.39
LMOh7858_0383	lmo0343 (*tal2*)	LMRG_00034	Transaldolase (EC 2.2.1.2)	3.98
LMOh7858_0384	lmo0344	LMRG_00035	oxidoreductase, short-chain dehydrogenase/reductase family	3.69
LMOh7858_0385	lmo0345	LMRG_00036	Ribose 5-phosphate isomerase B (EC 5.3.1.6)	5.86

[a]Strain EGD-e (GenBank accession no.: NC_003210) and 10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;
[b]FC, fold change, FC= average NRC(CSS) ÷ average NRC(MBHIB); only genes with FDR <0.05 are listed.

Supplemental Table S4.6. Upregulated genes involved in agmatine deiminase in H7858 grown on CSS compared to in MBHIB at 7°C

Gene name in			H7858 gene product	FC[b]
H7858	EGD-e[a]	10403S[a]		
LMOh7858_0048	lmo0036 (*arcB*)	LMRG_02465 (arcB)	Putrescine carbamoyltransferase (EC 2.1.3.6)	31.36
LMOh7858_0049	lmo0037	LMRG_02466	Agmatine/putrescine antiporter, associated with agmatine catabolism	19.56
LMOh7858_0050	lmo0038 (*aguA1*)	LMRG_02467	Agmatine deiminase (EC 3.5.3.12)	13.67
LMOh7858_0051	lmo0039	LMRG_02468	Carbamate kinase (EC 2.7.2.2)	18.29

[a]Strain EGD-e (GenBank accession no.: NC_003210) and 10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;
[b]FC, fold change, FC= average NRC(CSS) ÷ average NRC(MBHIB); only genes with FDR <0.05 are listed

Supplemental Table S4.7. Upregulated genes that are regulated by PrfA in H7858 grown on CSS compared to in MBHIB at 7°C

Gene name in			H7858 gene product	FC[b]
H7858	EGD-e[a]	10403S[a]		
LMOh7858_0499	lmo0434 (*inlB*)	LMRG_00127	Internalin B (GW modules)	2.71
LMOh7858_0221	lmo0201 (*plcA*)	LMRG_02623	Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	2.83
LMOh7858_0222	lmo0202 (*hly*)	LMRG_02624	Thiol-activated cytolysin	2.54
LMOh7858_0224	lmo0204 (*actA*)	LMRG_02626	Actin-assembly inducing protein ActA precursor	2.86
LMOh7858_0225	lmo0205 (*plcB*)	LMRG_02627	Broad-substrate range phospholipase C (EC 3.1.4.3)	3.19

[a]Strain EGD-e (GenBank accession no.: NC_003210) and 10403S (GenBank accession no.: NC_017544) are *L. monocytogenes*;

[b]FC, fold change, FC= average NRC(CSS) ÷ average NRC(MBHIB); only genes with FDR <0.05 are listed.